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ARRAYS DEVICES AND METHODS OF USE THEREOF

This application is a continuation-in-part of U.S. Patent Application No. 09/353,555, filed on July 14, 1999, which is a continuation-in-part of U.S. Patent Application No. 09/115,455, filed July 14, 1998, both of which are herein incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION**Field of the Invention**

The present invention relates generally to arrays of protein-capture agents and methods for the parallel detection and analysis of up to a large number of proteins in a sample. More specifically, the present invention relates to proteomics and the measurement of gene activity at the protein level in cells.

Description of Related Art

1 Although attempts to evaluate gene activity and to decipher biological processes
2 including those of disease processes and drug effects have traditionally focused on
3 genomics, proteomics offers a more direct and promising look at the biological functions of
4 a cell. Proteomics involves the qualitative and quantitative measurement of gene activity by
5 detecting and quantitating expression at the protein level, rather than at the messenger RNA
6 level. Proteomics also involves the study of non-genome encoded events including the post-
7 translational modification of proteins, interactions between proteins, and the location of
8 proteins within the cell. The structure, function, or level of activity of the proteins expressed
9 by a cell are also of interest. Essentially, proteomics involves the study of part or all of the
10 status of the total protein contained within or secreted by a cell.

11 The study of gene expression at the protein level is important because many of the
12 most important cellular processes are regulated by the protein status of the cell, not by the
13 status of gene expression. Also, the protein content of a cell is highly relevant to drug
14 discovery efforts since most drugs are designed to be active against protein targets.

15 Measuring the mRNA abundances of a cell provides only an indirect and incomplete
16 assessment of the protein content of a cell. The level of active protein that is produced in a
17 cell is often determined by factors other than the amount of mRNA produced. For instance,
18 both protein maturation and protein degradation are actively controlled in the cell and a
19 protein's activity status can be regulated by post-translational modifications. Studies
20 comparing mRNA transcript abundances to protein abundances have found only a limited
21 correlation (coefficient of about 0.43-0.48) between the two (Anderson and Anderson,
22 *Electrophoresis*, 19:1853-1861, 1998). Furthermore, the extreme lability of RNA in samples

1 due to chemical and enzymatic degradation makes the evaluation of genetic expression at
2 the protein level more practical than at the mRNA level.

3 Current technologies for the analysis of proteomes are based on a variety of protein
4 separation techniques followed by identification of the separated proteins. The most popular
5 method is based on 2D-gel electrophoresis followed by "in-gel" proteolytic digestion and
6 mass spectroscopy. Alternatively, Edman methods may be used for the sequencing. This
7 2D-gel technique requires large sample sizes, is time consuming, and is currently limited in
8 its ability to reproducibly resolve a significant fraction of the proteins expressed by a human
9 cell. Techniques involving some large-format 2D-gels can produce gels which separate a
10 larger number of proteins than traditional 2D-gel techniques, but reproducibility is still poor
11 and over 95% of the spots cannot be sequenced due to limitations with respect to sensitivity
12 of the available sequencing techniques. The electrophoretic techniques are also plagued by a
13 bias towards proteins of high abundance.

14 Standard assays for the presence of an analyte in a solution, such as those commonly
15 used for diagnostics, for instance, involve the use of an antibody which has been raised
16 against the targeted antigen. Multianalyte assays known in the art involve the use of
17 multiple antibodies and are directed towards assaying for multiple analytes. However, these
18 multianalyte assays have not been directed towards assaying the total or partial protein
19 content of a cell or cell population. Furthermore, sample sizes required to adapt such
20 standard antibody assay approaches to the analysis of even a fraction of the estimated
21 100,000 or more different proteins of a human cell and their various modified states are
22 prohibitively large. Automation and/or miniaturization of antibody assays are required if

1 large numbers of proteins are to be assayed simultaneously. Materials, surface coatings, and
2 detection methods used for macroscopic immunoassays and affinity purification are not
3 readily transferable to the formation or fabrication of miniaturized protein arrays.

4 Miniaturized DNA chip technologies have been developed (for example, see U.S.
5 Patent Nos. 5,412,087, 5,445,934, and 5,744,305) and are currently being exploited for the
6 screening of gene expression at the mRNA level. These chips can be used to determine
7 which genes are expressed by different types of cells and in response to different conditions.

8 However, DNA biochip technology is not transferable to protein-binding assays such as
9 antibody assays because the chemistries and materials used for DNA biochips are not readily
10 transferable to use with proteins. Nucleic acids such as DNA withstand temperatures up to
11 100°C, can be dried and re-hydrated without loss of activity, and can be bound physically or
12 chemically directly to organic adhesion layers supported by materials such as glass while
13 maintaining their activity. In contrast, proteins such as antibodies are preferably kept
14 hydrated and at ambient temperatures are sensitive to the physical and chemical properties of
15 the support materials. Therefore, maintaining protein activity at the liquid-solid interface
16 requires entirely different immobilization strategies than those used for nucleic acids. The
17 proper orientation of the antibody or other protein at the interface is desirable to ensure
18 accessibility of their active sites with interacting molecules. With miniaturization of the
19 chip and decreased feature sizes, the ratio of accessible to non-accessible and the ratio of
20 active to inactive antibodies or proteins become increasingly relevant and important.

1 Thus, there is a need for the ability to assay in parallel a multitude of proteins
2 expressed by a cell or a population of cells in an organism, including up to the total set of
3 proteins expressed by the cell or cells.

4 SUMMARY OF THE INVENTION

5 The present invention is directed to arrays of protein-capture agents and methods of
6 use thereof that satisfy the need to assay in parallel a multitude of proteins expressed by a
7 cell or population of cells in an organism, including up to the total protein content of a cell.

8 In one embodiment, the present invention provides an array of
9 protein-capture agents comprising: a substrate; at least one organic thinfilm covering some
10 or all of the surface of the substrate; and a plurality of patches arranged in discrete, known
11 regions on the portions of the substrate surface covered by organic thinfilm, wherein (i) each
12 patch comprises protein-capture agents immobilized on the organic thinfilm, where the
13 protein-capture agents of a given patch are capable of binding a particular expression
14 product, or a fragment thereof, of a cell or population of cells in an organism; and (ii) the
15 array comprises a plurality of different protein-capture agents, each of which is capable of
16 binding a different expression product, or fragment thereof, of the cell or population of cells
17 in the organism.

18 In a second embodiment, the invention provides an array of bound proteins
19 which comprises both the array of protein-capture agents of the invention and a plurality of
20 different proteins which are expression products, or fragments thereof, of a cell or
21 population of cells in an organism, where each of the different proteins is bound to a protein-
22 capture agent on a separate patch of the array.

1 Methods of using the arrays of protein-capture agents of the invention are also
2 provided. In one embodiment of the invention, a method of assaying in parallel for a
3 plurality of different proteins in a sample which are expression products, or fragments
4 thereof, of a cell or a population of cells in an organism, is provided which comprises first
5 delivering the sample to the array of protein-capture agents of the invention under conditions
6 suitable for protein binding, wherein each of the proteins being assayed is a binding partner
7 of the protein-capture agent of at least one patch on the array. The final step comprises
8 detecting, either directly or indirectly, for the presence or amount of protein bound to each
9 patch of the array. This method optionally further comprises the step of further
10 characterizing the proteins bound to at least one patch of the array.

11 In another embodiment of the invention, a method for determining the protein
12 expression pattern of a cell or a population of cells in an organism is provided which
13 comprises first delivering a sample containing the expression products, or fragments thereof,
14 of the cell or population of cells to the array of protein-capture agents of the invention under
15 conditions suitable for protein binding. The final step comprises detecting, either directly or
16 indirectly, for the presence or amount of protein bound to each patch of the array. In an
17 alternative embodiment, a similar method for comparing the protein expression patterns of
18 two cells or populations of cells is also provided.

19 In still another embodiment of the invention, an alternative method of assaying in
20 parallel for a plurality of different proteins in a sample which are expression products, or
21 fragments thereof, of a cell or a population of cells in an organism is provided. The method
22 of this embodiment comprises first contacting the sample with an array of spatially distinct

1 patches of different protein-capture agents under conditions suitable for protein binding,
2 wherein each of the proteins being assayed is a binding partner of the protein-capture agent
3 of at least one patch on the array. The last step of the method involves detecting, either
4 directly or indirectly, for the presence or amount of protein bound to each patch of the array.

5 In a still further embodiment, a method of producing an array of protein-capture
6 agents is provided which comprises the following steps: selecting protein-capture agents
7 from a library of protein-capture agents, wherein the protein-capture agents are selected by
8 their binding affinity to the proteins from a cellular extract or body fluid; producing a
9 plurality of purified samples of the selected protein-capture agents; and immobilizing the
10 protein-capture agent of each different purified sample onto an organic thinfilm on a separate
11 patch on the substrate surface to form a plurality of patches of protein-capture agents on
12 discrete, known regions of the surface of a substrate.

13 In an alternative embodiment, the invention provides a method for producing an
14 array of protein-capture agents which comprises a first step of selecting protein-capture
15 agents from a library of protein-capture agents, wherein the protein-capture agents are
16 selected by their binding affinity to proteins which are the expression products, or fragments
17 thereof, of a cDNA expression library. The second step of the method comprises producing
18 a plurality of purified samples of the protein-capture agents selected in the first step. The
19 third step comprises immobilizing the protein-capture agent of each different purified
20 sample onto an organic thinfilm on a separate patch on the substrate surface to form a
21 plurality of patches of protein-capture agents on discrete, known regions of the surface of a
22 substrate.

1 In another aspect, the invention provides for array devices for use in analyzing
2 molecular events between one or more biomolecules and one or more analytes comprising: a
3 substrate having at least one surface; one or more immobilization regions formed on the
4 known regions of the surface(s), wherein the immobilization regions are adapted for
5 attaching the biomolecules to the surface; and, one or more border regions formed on the
6 surface surrounding the immobilization regions, the border region(s) having a first wettable
7 state and a selectively achievable second wettable state different from the first wettable state.

8 In preferred embodiments, such array devices may further include one or more convertible
9 functional groups adapted for selectively converting between a first wettable form and a
10 second wettable form when activated by to impart upon the border regions the second
11 wettable state from the first wettable state; such convertible functional groups may be
12 activated by an activity selected from the group consisting of photocleavage, photo-
13 isomerization, catalytic-polymerization, and photoreaction activities; such convertible
14 functional groups may further have a first wettable state moiety attached to the surface
15 through at least one of the convertible functional groups, wherein the first wettable state
16 moiety imparts the first wettable state upon the border regions, and whereby removal of the
17 first wettable state moiety from the convertible functional groups causes the border regions
18 to attain the second wettable state; such first wettable state moiety may be a dendrimer or
19 dendritic molecule; and the immobilization regions may further comprise biomolecules
20 immobilized within the immobilization regions.

21 In another aspect, the invention further provides for methods for making an array of
22 one or more biomolecules for use in analyzing molecular events between one or more of the

1 biomolecules and one or more analytes comprising: providing the array device comprising: a
2 substrate having at least one surface; one or more immobilization regions formed on the
3 known regions of the surface(s), wherein the immobilization regions are adapted for
4 attaching the biomolecules to the surface; and, one or more border regions formed on the
5 surface surrounding the immobilization regions, the border region(s) having a first wettable
6 state and a selectively achievable second wettable state different from the first wettable state;
7 depositing a first liquid containing at least one of the biomolecules onto at least one selected
8 immobilization region such that the first liquid deposited is maintained within the selected
9 region in-part by the first wettable state of at least one of the border regions; allowing at least
10 one of the biomolecules contained in the deposited first liquid to attach to the surface within
11 the selected immobilization region; removing the first liquid from the selected
12 immobilization region; and activating the border region(s) partly or wholly maintaining the
13 first liquid within the selected immobilization regions such that such border regions partly or
14 wholly maintaining the liquid within the selected immobilization regions no longer are
15 capable of maintaining the first liquid, or a second liquid within the selected immobilization
16 region.

17 Yet another aspect of the invention provides for methods of carrying out a molecular
18 reaction on a surface of an array comprising the steps of: providing the array device
19 comprising: a substrate having at least one surface; one or more reaction regions formed on
20 the known regions of the surface(s), wherein the reaction regions are adapted for reacting
21 molecules adjacent the surface; and, one or more border regions formed on the surface
22 surrounding the reaction regions, the border region(s) having a first wettable state and a

1 selectively achievable second wettable state different from the first wettable state; depositing
2 a first liquid containing at least one of the molecules onto at least one selected reaction
3 region such that the first liquid deposited is maintained within the selected reaction region
4 in-part by the first wettable state of at least one of the border regions; allowing at least one of
5 the biomolecules contained in the deposited first liquid to contact the surface within the
6 selected reaction regions; removing the first liquid from the selected reaction regions;
7 activating the border region(s) partly or wholly maintaining the first liquid within the
8 selected reaction regions such that the border regions partly or wholly maintaining the liquid
9 within the selected reaction regions no longer are capable of maintaining the first liquid, or a
10 second liquid within the selected reaction regions.

11 12 BRIEF DESCRIPTION OF THE FIGURES

13 Figure 1 shows the top view of an array of patches reactive towards protein-capture
14 agents.

15 Figure 2 shows the cross section of an individual patch of the array of Figure 1.

16 Figure 3 shows the cross section of a row of monolayer-covered patches of the array
17 of Figure 1.

18 Figure 4 shows a thiolreactive monolayer on a substrate.

19 Figure 5 shows an aminoreactive monolayer on a coated substrate.

20 Figure 6 shows the immobilization of a protein-capture agent on a monolayer-coated
21 substrate via an affinity tag.

1 Figure 7 shows the immobilization of a protein-capture agent on a monolayer-coated
2 substrate via an affinity tag and an adaptor.

3 Figure 8 shows a schematic of a fluorescence detection unit which may be used to
4 monitor binding of proteins by the protein-capture agents of the array.

5 Figure 9 shows a schematic of an ellipsometric detection unit which may be used to
6 monitor binding of proteins by the protein-capture agents of the array.

7 Figure 10 shows several panels which depict the different stages of an array having
8 two different border region wettable states.

9 DETAILED DESCRIPTION OF THE INVENTION

10 A variety of arrays of protein-capture agents and methods useful for multianalyte
11 analyses and analyses of protein expression and modification in cells are provided by the
12 present invention.

13 (a) Definitions.

14 The term "protein-capture agent" means a molecule or a multi-molecular complex
15 which can bind a protein to itself. Protein-capture agents preferably bind their binding
16 partners in a substantially specific manner. Protein-capture agents with a dissociation
17 constant (K_D) of less than about 10^{-6} are preferred. The protein-capture agent will most
18 typically be a biomolecule such as a protein or a polynucleotide. The biomolecule may
19 optionally be a naturally occurring, recombinant, or synthetic biomolecule. Antibodies or
20 antibody fragments are highly suitable as protein-capture agents. Antigens may also serve as
21 protein-capture agents, since they are capable of binding antibodies. A receptor which binds
22

1 a protein ligand is another example of a possible protein-capture agent. For instance,
2 protein-capture agents are understood not to be limited to agents which only interact with
3 their binding partners through noncovalent interactions. Protein-capture agents may also
4 optionally become covalently attached to proteins which they bind. For instance, the
5 protein-capture agent may be photocrosslinked to its binding partner following binding.

6 The term "binding partner" means a protein which is bound by a particular protein-
7 capture agent, preferably in a substantially specific manner. In some cases, the protein-
8 capture agent may be a cellular or extracellular protein and the binding partner may be the
9 entity normally bound *in vivo*. In other embodiments, however, the binding partner may be
10 the protein or peptide on which the protein-capture agent was selected (through *in vitro* or *in*
11 *vivo* selection) or raised (as in the case of antibodies). A binding partner may be shared by
12 more than one protein-capture agent. For instance, a binding partner which is bound by a
13 variety of polyclonal antibodies may bear a number of different epitopes. One protein-
14 capture agent may also bind to a multitude of binding partners, for instance, if the binding
15 partners share the same epitope.

16 A "protein" means a polymer of amino acid residues linked together by peptide
17 bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size,
18 structure, or function. Typically, however, a protein will be at least six amino acids long.
19 Preferably, if the protein is a short peptide, it will be at least about 10 amino acid residues
20 long. A protein may be naturally occurring, recombinant, or synthetic, or any combination
21 of these. A protein may also be just a fragment of a naturally occurring protein or peptide. A
22 protein may be a single molecule or may be a multi-molecular complex. The term protein

1 may also apply to amino acid polymers in which one or more amino acid residues is an
2 artificial chemical analogue of a corresponding naturally occurring amino acid. An amino
3 acid polymer in which one or more amino acid residues is an “unnatural” amino acid, not
4 corresponding to any naturally occurring amino acid, is also encompassed by the use of the
5 term “protein” herein.

6 A “fragment of a protein” means a protein which is a portion of another protein. For
7 instance, fragments of a proteins may be a polypeptides obtained by digesting full-length
8 protein isolated from cultured cells. A fragment of a protein will typically comprise at least
9 six amino acids. More typically, the fragment will comprise at least ten amino acids.
10 Preferably, the fragment comprises at least about 16 amino acids.

11 An “expression product” is a biomolecule, such as a protein, which is produced when
12 a gene in an organism is expressed. An expression product may optionally comprise post-
13 translational modifications.

14 The term “antibody” means an immunoglobulin, whether natural or partially or
15 wholly synthetically produced. All derivatives thereof which maintain specific binding
16 ability are also included in the term. The term also covers any protein having a binding
17 domain which is homologous or largely homologous to an immunoglobulin binding domain.
18 These proteins may be derived from natural sources, or partly or wholly synthetically
19 produced. An antibody may be monoclonal or polyclonal. The antibody may be a member
20 of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and
21 IgE. Derivatives of the IgG class, however, are preferred in the present invention.

1 The term “antibody fragment” refers to any derivative of an antibody which is less
2 than full-length. Preferably, the antibody fragment retains at least a significant portion of the
3 full-length antibody’s specific binding ability. Examples of antibody fragments include, but
4 are not limited to, Fab, Fab’, F(ab’)₂, scFv, Fv, dsFv diabody, and Fd fragments. The
5 antibody fragment may be produced by any means. For instance, the antibody fragment may
6 be enzymatically or chemically produced by fragmentation of an intact antibody or it may be
7 recombinantly produced from a gene encoding the partial antibody sequence. Alternatively,
8 the antibody fragment may be wholly or partially synthetically produced. The antibody
9 fragment may optionally be a single chain antibody fragment. Alternatively, the fragment
10 may comprise multiple chains which are linked together, for instance, by disulfide linkages.
11 The fragment may also optionally be a multimolecular complex. A functional antibody
12 fragment will typically comprise at least about 50 amino acids and more typically will
13 comprise at least about 200 amino acids.

14 Single-chain Fvs (scFvs) are recombinant antibody fragments consisting of only the
15 variable light chain (V_L) and variable heavy chain (V_H) covalently connected to one another
16 by a polypeptide linker. Either V_L or V_H may be the NH₂-terminal domain. The polypeptide
17 linker may be of variable length and composition so long as the two variable domains are
18 bridged without serious steric interference. Typically, the linkers are comprised primarily of
19 stretches of glycine and serine residues with some glutamic acid or lysine residues
20 interspersed for solubility.

21 “Diabodies” are dimeric scFvs. The components of diabodies typically have shorter
22 peptide linkers than most scFvs and they show a preference for associating as dimers.

1 An "Fv" fragment consists of one V_H and one V_L domain held together by
2 noncovalent interactions. The term "dsFv" is used herein to refer to an Fv with an
3 engineered intermolecular disulfide bond to stabilize the V_H - V_L pair.

4 A "F(ab')₂" fragment is an antibody fragment essentially equivalent to that obtained
5 from immunoglobulins (typically IgG) by digestion with an enzyme pepsin at pH 4.0-4.5.
6 The fragment may be recombinantly produced.

7 A "Fab'" fragment is an antibody fragment essentially equivalent to that obtained by
8 reduction of the disulfide bridge or bridges joining the two heavy chain pieces in the F(ab')₂
9 fragment. The Fab' fragment may be recombinantly produced.

10 A "Fab" fragment is an antibody fragment essentially equivalent to that obtained by
11 digestion of immunoglobulins (typically IgG) with the enzyme papain. The Fab fragment
12 may be recombinantly produced. The heavy chain segment of the Fab fragment is the Fd
13 piece.

14 A "population of cells in an organism" means a collection of more than one cell in a
15 single organism or more than one cell originally derived from a single organism. The cells in
16 the collection are preferably all of the same type. They may all be from the same tissue in an
17 organism, for instance. Most preferably, gene expression in all of the cells in the population
18 is identical or nearly identical.

19 "Conditions suitable for protein binding" means those conditions (in terms of salt
20 concentration, pH, detergent, protein concentration, temperature, etc.) which allow for
21 binding to occur between an immobilized protein-capture agent and its binding partner in

1 solution. Preferably, the conditions are not so lenient that a significant amount of
2 nonspecific protein binding occurs.

3 A "body fluid" may be any liquid substance extracted, excreted, or secreted from an
4 organism or tissue of an organism. The body fluid need not necessarily contain cells. Body
5 fluids of relevance to the present invention include, but are not limited to, whole blood,
6 serum, urine, plasma, cerebral spinal fluid, tears, sinovial fluid, and amniotic fluid.

7 An "array" is an arrangement of entities in a pattern on a substrate. Although the
8 pattern is typically a two-dimensional pattern, the pattern may also be a three-dimensional
9 pattern.

10 A "patch of protein-capture agents" means a discrete region of immobilized protein-
11 capture agents on the surface of a substrate. The patches may be of any geometric shape or
12 may be irregularly shaped. For instance, the patch may be, but need not necessarily be,
13 square in shape.

14 "Proteomics" means the study of or the characterization of either the proteome or
15 some fraction of the proteome. The "proteome" is the total collection of the intracellular
16 proteins of a cell or population of cells and the proteins secreted by the cell or population of
17 cells. This characterization most typically includes measurements of the presence, and
18 usually quantity, of the proteins which have been expressed by a cell. The function,
19 structural characteristics (such as post translational modification), and location within the
20 cell of the proteins may also be studied. "Functional proteomics" refers to the study of the
21 functional characteristics, activity level, and structural characteristics of the protein
22 expression products of a cell or population of cells.

1 The term “substrate” refers to the bulk, underlying, and core material of the arrays of
2 the invention.

3 The terms “micromachining” and “microfabrication” both refer to any number of
4 techniques which are useful in the generation of microstructures (structures with feature
5 sizes of sub-millimeter scale). Such technologies include, but are not limited to, laser
6 ablation, electrodeposition, physical and chemical vapor deposition, photolithography, and
7 wet chemical and dry etching. Related technologies such as injection molding and LIGA (X-
8 ray lithography, electrodeposition, and molding) are also included. Most of these techniques
9 were originally developed for use in semiconductors, microelectronics, and Micro-
10 ElectroMechanical Systems (MEMS) but are applicable to the present invention as well.

11 The term “coating” means a layer that is either naturally or synthetically formed on or
12 applied to the surface of the substrate. For instance, exposure of a substrate, such as silicon,
13 to air results in oxidation of the exposed surface. In the case of a substrate made of silicon, a
14 silicon oxide coating is formed on the surface upon exposure to air. In other instances, the
15 coating is not derived from the substrate and may be placed upon the surface via mechanical,
16 physical, electrical, or chemical means. An example of this type of coating would be a metal
17 coating that is applied to a silicon or polymer substrate or a silicon nitride coating that is
18 applied to a silicon substrate. Although a coating may be of any thickness, typically the
19 coating has a thickness smaller than that of the substrate.

20 An “interlayer” is an additional coating or layer that is positioned between the first
21 coating and the substrate. Multiple interlayers may optionally be used together. The primary
22 purpose of a typical interlayer is to aid adhesion between the first coating and the substrate.

1 One such example is the use of a titanium or chromium interlayer to help adhere a gold
2 coating to a silicon or glass surface. However, other possible functions of an interlayer are
3 also anticipated. For instance, some interlayers may perform a role in the detection system
4 of the array (such as a semiconductor or metal layer between a nonconductive substrate and a
5 nonconductive coating).

6 An "organic thinfilm" is a thin layer of organic molecules which has been applied to
7 a substrate or to a coating on a substrate if present. Typically, an organic thinfilm is less
8 than about 20 nm thick. Optionally, an organic thinfilm may be less than about 10 nm thick.

9 An organic thinfilm may be disordered or ordered. For instance, an organic thinfilm can be
10 amorphous (such as a chemisorbed or spin-coated polymer) or highly organized (such as a
11 Langmuir-Blodgett film or self-assembled monolayer). An organic thinfilm may be
12 heterogeneous or homogeneous. Organic thinfilms which are monolayers are preferred. A
13 lipid bilayer or monolayer is a preferred organic thinfilm. Optionally, the organic thinfilm
14 may comprise a combination of more than one form of organic thinfilm. For instance, an
15 organic thinfilm may comprise a lipid bilayer on top of a self-assembled monolayer. A
16 hydrogel may also compose an organic thinfilm. The organic thinfilm will typically have
17 functionalities exposed on its surface which serve to enhance the surface conditions of a
18 substrate or the coating on a substrate in any of a number of ways. For instance, exposed
19 functionalities of the organic thinfilm are typically useful in the binding or covalent
20 immobilization of the protein-capture agents to the patches of the array. Alternatively, the
21 organic thinfilm may bear functional groups (such as polyethylene glycol (PEG)) which
22 reduce the non-specific binding of molecules to the surface. Other exposed functionalities

1 serve to tether the thinfilm to the surface of the substrate or the coating. Particular
2 functionalities of the organic thinfilm may also be designed to enable certain detection
3 techniques to be used with the surface. Alternatively, the organic thinfilm may serve the
4 purpose of preventing inactivation of a protein-capture agent or the protein to be bound by a
5 protein-capture agent from occurring upon contact with the surface of a substrate or a
6 coating on the surface of a substrate.

7 A "monolayer" is a single-molecule thick organic thinfilm. A monolayer may be
8 disordered or ordered. A monolayer may optionally be a polymeric compound, such as a
9 polynonionic polymer, a polyionic polymer, or a block-copolymer. For instance, the
10 monolayer may be composed of a poly(amino acid) such as polylysine. A monolayer which
11 is a self-assembled monolayer, however, is most preferred. One face of the self-assembled
12 monolayer is typically composed of chemical functionalities on the termini of the organic
13 molecules that are chemisorbed or physisorbed onto the surface of the substrate or, if
14 present, the coating on the substrate if present. Examples of suitable functionalities of
15 monolayers include the positively charged amino groups of poly-L-lysine for use on
16 negatively charged surfaces and thiols for use on gold surfaces. Typically, the other face of
17 the self-assembled monolayer is exposed and may bear any number of chemical
18 functionalities (end groups). Preferably, the molecules of the self-assembled monolayer are
19 highly ordered.

20 A "self-assembled monolayer" is a monolayer which is created by the spontaneous
21 assembly of molecules. The self-assembled monolayer may be ordered, disordered, or
22 exhibit short- to long-range order.

1 An “affinity tag” is a functional moiety capable of directly or indirectly immobilizing
2 a protein-capture agent onto an exposed functionality of the organic thinfilm. Preferably, the
3 affinity tag enables the site-specific immobilization and thus enhances orientation of the
4 protein-capture agent onto the organic thinfilm. In some cases, the affinity tag may be a
5 simple chemical functional group. Other possibilities include amino acids, poly(amino acid)
6 tags, or full-length proteins. Still other possibilities include carbohydrates and nucleic acids.
7 For instance, the affinity tag may be a polynucleotide which hybridizes to another
8 polynucleotide serving as a functional group on the organic thinfilm or another
9 polynucleotide serving as an adaptor. The affinity tag may also be a synthetic chemical
10 moiety. If the organic thinfilm of each of the patches comprises a lipid bilayer or monolayer,
11 then a membrane anchor is a suitable affinity tag. The affinity tag may be covalently or
12 noncovalently attached to the protein-capture agent. For instance, if the affinity tag is
13 covalently attached to the protein-capture agent it may be attached via chemical conjugation
14 or as a fusion protein. The affinity tag may also be attached to the protein-capture agent via
15 a cleavable linkage. Alternatively, the affinity tag may not be directly in contact with the
16 protein-capture agent. The affinity tag may instead be separated from the protein-capture
17 agent by an adaptor. The affinity tag may immobilize the protein-capture agent to the
18 organic thinfilm either through noncovalent interactions or through a covalent linkage.

19 An “adaptor”, for purposes of this invention, is any entity that links an affinity tag to
20 the protein-capture agent. The adaptor may be, but need not necessarily be, a discrete
21 molecule that is noncovalently attached to both the affinity tag and the protein-capture agent.
22 The adaptor can instead be covalently attached to the affinity tag or the protein-capture agent

1 or both (via chemical conjugation or as a fusion protein, for instance). Proteins such as full-
2 length proteins, polypeptides, or peptides are typical adaptors. Other possible adaptors
3 include carbohydrates or nucleic acids.

4 The term “fusion protein” refers to a protein composed of two or more polypeptides
5 that, although typically unjoined in their native state, are joined by their respective amino
6 and carboxyl termini through a peptide linkage to form a single continuous polypeptide. It is
7 understood that the two or more polypeptide components can either be directly joined or
8 indirectly joined through a peptide linker/spacer.

9 The term “normal physiological condition” means conditions that are typical inside a
10 living organism or a cell. While it is recognized that some organs or organisms provide
11 extreme conditions, the intra-organismal and intra-cellular environment normally varies
12 around pH 7 (*i.e.*, from pH 6.5 to pH 7.5), contains water as the predominant solvent, and
13 exists at a temperature above 0°C and below 50°C. It will be recognized that the
14 concentration of various salts depends on the organ, organism, cell, or cellular compartment
15 used as a reference.

16
17 (b) Arrays of the invention.

18 The present invention is directed to arrays of protein-capture agents which can bind a
19 plurality of proteins that are the expression products, or fragments thereof, of a cell or
20 population of cells in an organism and therefore can be used to evaluate gene expression at
21 the protein level. Typically, the arrays comprise micrometer-scale, two-dimensional patterns

1 of patches of protein-capture agents immobilized on an organic thinfilm coating on the
2 surface of the substrate.

3 In one embodiment of the invention, the array of protein-capture agents comprises a
4 substrate, at least one organic thinfilm covering some or all of the surface of the substrate,
5 and a plurality of patches arranged in discrete, known regions on the portions of the substrate
6 surface covered by organic thinfilm, wherein (i) each patch comprises protein-capture agents
7 immobilized on the organic thinfilm, wherein said protein-capture agents of a given patch
8 are capable of binding a particular expression product, or a fragment thereof, of a cell or
9 population of cells in an organism, and (ii) the array comprises a plurality of different
10 protein-capture agents, each of which is capable of binding a different expression product, or
11 fragment thereof, of the cell or population of cells.

12 The protein-capture agents are preferably covalently immobilized on the
13 patches of the array, either directly or indirectly.

14 In most cases, the array will comprise at least about ten patches. In a preferred
15 embodiment, the array comprises at least about 50 patches. In a particularly preferred
16 embodiment the array comprises at least about 100 patches. In alternative preferred
17 embodiments, the array of protein-capture agents may comprise more than 10^3 , 10^4 or 10^5
18 patches.

19 The area of surface of the substrate covered by each of the patches is preferably no
20 more than about 0.25 mm^2 . Preferably, the area of the substrate surface covered by each of
21 the patches is between about $1 \text{ }\mu\text{m}^2$ and about $10,000 \text{ }\mu\text{m}^2$. In a particularly preferred
22 embodiment, each patch covers an area of the substrate surface from about $100 \text{ }\mu\text{m}^2$ to about

1 2,500 μm^2 . In an alternative embodiment, a patch on the array may cover an area of the
2 substrate surface as small as about 2,500 nm^2 , although patches of such small size are
3 generally not necessary for the use of the array .

4 The patches of the array may be of any geometric shape. For instance, the patches
5 may be rectangular or circular. The patches of the array may also be irregularly shaped. The
6 patches are optionally elevated from the median plan of the underlying substrate.

7 The distance separating the patches of the array can vary. Preferably, the patches of
8 the array are separated from neighboring patches by about 1 μm to about 500 μm . Typically,
9 the distance separating the patches is roughly proportional to the diameter or side length of
10 the patches on the array if the patches have dimensions greater than about 10 μm . If the
11 patch size is smaller, then the distance separating the patches will typically be larger than the
12 dimensions of the patch.

13 In a preferred embodiment of the array, the patches of the array are all contained
14 within an area of about 1 cm^2 or less on the surface of the substrate. In one preferred
15 embodiment of the array, therefore, the array comprises 100 or more patches within a total
16 area of about 1 cm^2 or less on the surface of the substrate. Alternatively, a particularly
17 preferred array comprises 10^3 or more patches within a total area of about 1 cm^2 or less. A
18 preferred array may even optionally comprise 10^4 or 10^5 or more patches within an area of
19 about 1 cm^2 or less on the surface of the substrate. In other embodiments of the invention,
20 all of the patches of the array are contained within an area of about 1 mm^2 or less on the
21 surface of the substrate.

1 Typically, only one type of protein-capture agent is present on a single patch of the
2 array. If more than one type of protein-capture agent is present on a single patch, all of the
3 protein-capture agents of that patch must share a common binding partner. For instance, a
4 patch may comprise a variety of polyclonal antibodies to the same antigen (although,
5 potentially, the antibodies may bind different epitopes on that same antigen).

6 The arrays of the invention can have any number of a plurality of different protein-
7 capture agents. Typically the array comprises at least about ten different protein-capture
8 agents. Preferably, the array comprises at least about 50 different protein-capture agents.
9 More preferably, the array comprises at least about 100 different protein-capture agents.
10 Alternative preferred arrays comprise more than about 10^3 different protein-capture agents or
11 more than about 10^4 different protein-capture agents. The array may even optionally
12 comprise more than about 10^5 different protein-capture agents.

13 The number of different protein-capture agents on the array will vary depending on
14 the application desired. For instance, if the array is to be used as a diagnostic tool in
15 evaluating the status of a tumor or other diseased tissue in a patient, an array comprising less
16 than about 100 different protein-capture agents may suffice since the necessary binding
17 partners of the protein-capture agent on the array are limited to only those proteins whose
18 expression is known to be indicative of the disease condition. However, if the array is to be
19 used to measure a significant portion of the total protein content of a cell, then the array
20 preferably comprises at least about 10,000 different protein-capture agents. Alternatively, a
21 more limited proteomics study, such as a study of the abundances of various human

1 transcription factors, for instance, might only require an array of about 100 different protein-
2 capture agents.

3 In one embodiment of the array, each of the patches of the array comprises a different
4 protein-capture agent. For instance, an array comprising about 100 patches could comprise
5 about 100 different protein-capture agents. Likewise, an array of about 10,000 patches could
6 comprise about 10,000 different protein-capture agents. In an alternative embodiment,
7 however, each different protein-capture agent is immobilized on more than one separate
8 patch on the array. For instance, each different protein-capture agent may optionally be
9 present on two to six different patches. An array of the invention, therefore, may comprise
10 about three-thousand protein-capture agent patches, but only comprise about one thousand
11 different protein-capture agents since each different protein-capture agent is present on three
12 different patches.

13 Typically, the number of different proteins which can be bound by the plurality of
14 different protein-capture agents on the array will be at least about ten. However, it is
15 preferred that the plurality of different protein-capture agents on the array is capable of
16 binding a higher number of different proteins, such as at least about 50 or at least about 100.
17 In still further preferred embodiments, the plurality of different proteins on the array is
18 capable of binding at least about 10^3 proteins. For some applications, such as those where it
19 is desirable to assay the entire protein content of a cell, or a significant fraction thereof, an
20 array where the plurality of protein-capture agents is capable of binding at least about 10^4
21 different proteins or even at least about 10^5 different proteins is most preferred.

1 In one embodiment of the invention, the binding partners of the plurality of protein-
2 capture agents on the array are proteins which are all expression products, or fragments
3 thereof, of a cell or population of cells of a single organism. The expression products may
4 be proteins, including peptides, of any size or function. They may be intracellular proteins
5 or extracellular proteins. The expression products may be from a one-celled or multicellular
6 organism. The organism may be a plant or an animal. In a preferred embodiment of the
7 invention, the binding partners are human expression products, or fragments thereof.

8 In one embodiment of the invention, the binding partners of the protein-capture
9 agents of the array may be a randomly chosen subset of all the proteins, including peptides,
10 which are expressed by a cell or population of cells in a given organism or a subset of all the
11 fragments of those proteins. Thus, the binding partners of the protein-capture agents of the
12 array optionally represent a wide distribution of different proteins from a single organism.

13 The binding partners of some or all of the protein-capture agents on the array need
14 not necessarily be known. The binding partner of a protein-capture agent of the array may
15 be a protein or peptide of unknown function. For instance, the different protein-capture
16 agents of the array may together bind a wide range of cellular proteins from a single cell
17 type, many of which are of unknown identity and/or function.

18 In another embodiment of the present invention, the binding partners of the protein-
19 capture agents on the array are related proteins. The different proteins bound by the protein-
20 capture agents may optionally be members of the same protein family. The different binding
21 partners of the protein-capture agents of the array may be either functionally related or just
22 suspected of being functionally related. The different proteins bound by the protein-capture

1 agents of the array may also be proteins which share a similarity in structure or sequence or
2 are simply suspected of sharing a similarity in structure or sequence. For instance, the
3 binding partners of the protein-capture agents on the array may optionally all be growth
4 factor receptors, hormone receptors, neurotransmitter receptors, catecholamine receptors,
5 amino acid derivative receptors, cytokine receptors, extracellular matrix receptors,
6 antibodies, lectins, cytokines, serpins, proteases, kinases, phosphatases, ras-like GTPases,
7 hydrolases, steroid hormone receptors, transcription factors, heat-shock transcription factors,
8 DNA-binding proteins, zinc-finger proteins, leucine-zipper proteins, homeodomain proteins,
9 intracellular signal transduction modulators and effectors, apoptosis-related factors, DNA
10 synthesis factors, DNA repair factors, DNA recombination factors, cell-surface antigens,
11 hepatitis C virus (HCV) proteases or HIV proteases.

12 In an alternative embodiment of the invention, the proteins which are the binding
13 partners of the protein-capture agents of the array may be fragments of the expression
14 products of a cell or population of cells in an organism.

15 A protein-capture agent on the array can be any molecule or complex of molecules
16 which has the ability to bind a protein and immobilize it to the site of the protein-capture
17 agent on the array. Preferably, the protein-capture agent binds its binding partner in a
18 substantially specific manner. Hence, the protein-capture agent may optionally be a protein
19 whose natural function in a cell is to specifically bind another protein, such as an antibody or
20 a receptor. Alternatively, the protein-capture agent may instead be a partially or wholly
21 synthetic or recombinant protein which specifically binds a protein. Alternatively, the
22 protein-capture agent may be a protein which has been selected *in vitro* from a mutagenized,

1 randomized, or completely random and synthetic library by its binding affinity to a specific
2 protein or peptide target. The selection method used may optionally have been a display
3 method such as ribosome display or phage display (see below). Alternatively, the protein-
4 capture agent obtained via *in vitro* selection may be a DNA or RNA aptamer which
5 specifically binds a protein target (for example: Potyrailo *et al.*, *Anal. Chem.*, **70**:3419-25,
6 1998; Cohen, *et al.*, *Proc. Natl. Acad. Sci. USA*, **95**:14272-7, 1998; Fukuda, *et al.*, *Nucleic*
7 *Acids Symp. Ser.*, (37):237-8, 1997). Alternatively, the *in vitro* selected protein-capture
8 agent may be a polypeptide (Roberts and Szostak, *Proc. Natl. Acad. Sci. USA*, **94**:12297-
9 302, 1997). In an alternative embodiment, the protein-capture agent may be a small
10 molecule which has been selected from a combinatorial chemistry library or is isolated from
11 an organism.

12 In a preferred embodiment of the array, however, the protein-capture agents are
13 proteins. In a particularly preferred embodiment, the protein-capture agents are antibodies or
14 antibody fragments. Although antibody moieties are exemplified herein, it is understood
15 that the present arrays and methods may be advantageously employed with other protein-
16 capture agents.

17 The antibodies or antibody fragments of the array may optionally be single-chain
18 Fvs, Fab fragments, Fab' fragments, F(ab')₂ fragments, Fv fragments, dsFvs diabodies, Fd
19 fragments, full-length, antigen-specific polyclonal antibodies, or full-length monoclonal
20 antibodies. In a preferred embodiment, the protein-capture agents of the array are
21 monoclonal antibodies, Fab fragments or single-chain Fvs.

1 The antibodies or antibody fragments may be monoclonal antibodies, even
2 commercially available antibodies, against known, well-characterized proteins.
3 Alternatively, the antibody fragments have been derived by selection from a library using the
4 phage display method. If the antibody fragments are derived individually by selection based
5 on binding affinity to known proteins, then, the binding partners of the antibody fragments
6 are known. In an alternative embodiment of the invention, the antibody fragments have been
7 derived by a phage display method comprising selection based on binding affinity to the
8 (typically, immobilized) proteins of a cellular extract or a body fluid. In this embodiment,
9 some or many of the antibody fragments of the array would bind proteins of unknown
10 identity and/or function.

11 Upon using the array of protein-capture agents to bind a plurality of expression
12 products, or fragments thereof, an array of bound proteins is created. Thus, another
13 embodiment of the invention provides an array of bound proteins which comprises (a) a
14 protein-capture agent array of the invention and (b) a plurality of different proteins which are
15 expression products, or fragments thereof, of a cell or a population of cells in an organism,
16 wherein each of the different proteins is bound to a protein-capture agent on a separate patch
17 of the array. Preferably, each of the different proteins is non-covalently bound to a protein-
18 capture agent.

19
20 (c) Substrates, coatings, and organic thinfilms.

21 The substrate of the array may be either organic or inorganic, biological or non-
22 biological, or any combination of these materials. In one embodiment, the substrate is

1 transparent or translucent. The portion of the surface of the substrate on which the patches
2 reside is preferably flat and firm or semi-firm. However, the array of the present invention
3 need not necessarily be flat or entirely two-dimensional. Significant topological features
4 may be present on the surface of the substrate surrounding the patches, between the patches
5 or beneath the patches. For instance, walls or other barriers may separate the patches of the
6 array.

7 Numerous materials are suitable for use as a substrate in the array embodiment of the
8 invention. For instance, the substrate of the invention array can comprise a material selected
9 from a group consisting of silicon, silica, quartz, glass, controlled pore glass, carbon,
10 alumina, titania, tantalum oxide, germanium, silicon nitride, zeolites, and gallium arsenide.
11 Many metals such as gold, platinum, aluminum, copper, titanium, and their alloys are also
12 options for substrates of the array. In addition, many ceramics and polymers may also be
13 used as substrates. Polymers which may be used as substrates include, but are not limited to,
14 the following: polystyrene; poly(tetra)fluoroethylene (PTFE); polyvinylidenedifluoride;
15 polycarbonate; polymethylmethacrylate; polyvinylethylene; polyethyleneimine;
16 poly(etherether)ketone; polyoxymethylene (POM); polyvinylphenol; polylactides;
17 polymethacrylimide (PMI); polyalkenesulfone (PAS); polypropylene, polyethylene;
18 polyhydroxyethylmethacrylate (HEMA); polydimethylsiloxane; polyacrylamide; polyimide;
19 and block-copolymers. Preferred substrates for the array include silicon, silica, glass, and
20 polymers. The substrate on which the patches reside may also be a combination of any of
21 the aforementioned substrate materials.

1 An array of the present invention may optionally further comprise a coating between
2 the substrate and the organic thinfilm of its patches. This coating may either be formed on
3 the substrate or applied to the substrate. The substrate can be modified with a coating by
4 using thin-film technology based, for instance, on physical vapor deposition (PVD), plasma-
5 enhanced chemical vapor deposition (PECVD), or thermal processing. Alternatively, plasma
6 exposure can be used to directly activate or alter the substrate and create a coating. For
7 instance, plasma etch procedures can be used to oxidize a polymeric surface (for example,
8 polystyrene or polyethylene to expose polar functionalities such as hydroxyls, carboxylic
9 acids, aldehydes and the like) which then acts as a coating.

10 The coating is optionally a metal film. Possible metal films include aluminum,
11 chromium, titanium, tantalum, nickel, stainless steel, zinc, lead, iron, copper, magnesium,
12 manganese, cadmium, tungsten, cobalt, and alloys or oxides thereof. In a preferred
13 embodiment, the metal film is a noble metal film. Noble metals that may be used for a
14 coating include, but are not limited to, gold, platinum, silver, and copper. In an especially
15 preferred embodiment, the coating comprises gold or a gold alloy. Electron-beam
16 evaporation may be used to provide a thin coating of gold on the surface of the substrate. In
17 a preferred embodiment, the metal film is from about 50 nm to about 500 nm in thickness.
18 In an alternative embodiment, the metal film is from about 1 nm to about 1 μ m in thickness.

19 In alternative embodiments, the coating comprises a composition selected from the
20 group consisting of silicon, silicon oxide, titania, tantalum oxide, silicon nitride, silicon
21 hydride, indium tin oxide, magnesium oxide, alumina, glass, hydroxylated surfaces, and
22 polymers.

1 In one embodiment of the invention array, the surface of the coating is atomically
2 flat. In this embodiment, the mean roughness of the surface of the coating is less than about
3 5 angstroms for areas of at least $25\ \mu\text{m}^2$. In a preferred embodiment, the mean roughness of
4 the surface of the coating is less than about 3 angstroms for areas of at least $25\ \mu\text{m}^2$. The
5 ultraflat coating can optionally be a template-stripped surface as described in Hegner *et al.*,
6 *Surface Science*, 1993, **291**:39-46 and Wagner *et al.*, *Langmuir*, 1995, **11**:3867-3875, both
7 of which are incorporated herein by reference.

8 It is contemplated that the coatings of many arrays will require the addition of at least
9 one adhesion layer between said coating and the substrate. Typically, the adhesion layer will
10 be at least 6 angstroms thick and may be much thicker. For instance, a layer of titanium or
11 chromium may be desirable between a silicon wafer and a gold coating. In an alternative
12 embodiment, an epoxy glue such as Epo-tek 377®, Epo-tek 301-2®, (Epoxy Technology
13 Inc., Billerica, Massachusetts) may be preferred to aid adherence of the coating to the
14 substrate. Determinations as to what material should be used for the adhesion layer would
15 be obvious to one skilled in the art once materials are chosen for both the substrate and
16 coating. In other embodiments, additional adhesion mediators or interlayers may be
17 necessary to improve the optical properties of the array, for instance, waveguides for
18 detection purposes.

19 Deposition or formation of the coating (if present) on the substrate is performed prior
20 to the formation of the organic thinfilm thereon. Several different types of coating may be
21 combined on the surface. The coating may cover the whole surface of the substrate or only
22 parts of it. The pattern of the coating may or may not be identical to the pattern of organic

1 thinfilms used to immobilize the protein-capture agents. In one embodiment of the
2 invention, the coating covers the substrate surface only at the site of the patches of protein-
3 capture agents. Techniques useful for the formation of coated patches on the surface of the
4 substrate which are organic thinfilm compatible are well known to those of ordinary skill in
5 the art. For instance, the patches of coatings on the substrate may optionally be fabricated by
6 photolithography, micromolding (PCT Publication WO 96/29629), wet chemical or dry
7 etching, or any combination of these.

8 The organic thinfilm on which each of the patches of protein-capture agents resides
9 forms a layer either on the substrate itself or on a coating covering the substrate. The
10 organic thinfilm on which the protein-capture agents of the patches are immobilized is
11 preferably less than about 20 nm thick. In some embodiments of the invention, the organic
12 thinfilm of each of the patches may be less than about 10 nm thick.

13 A variety of different organic thinfilms are suitable for use in the present invention.
14 Methods for the formation of organic thinfilms include *in situ* growth from the surface,
15 deposition by physisorption, spin-coating, chemisorption, self-assembly, or plasma-initiated
16 polymerization from gas phase. For instance, a hydrogel composed of a material such as
17 dextran can serve as a suitable organic thinfilm on the patches of the array. In one preferred
18 embodiment of the invention, the organic thinfilm is a lipid bilayer. In another preferred
19 embodiment, the organic thinfilm of each of the patches of the array is a monolayer. A
20 monolayer of polyarginine or polylysine adsorbed on a negatively charged substrate or
21 coating is one option for the organic thinfilm. Another option is a disordered monolayer of
22 tethered polymer chains. In a particularly preferred embodiment, the organic thinfilm is a

1 self-assembled monolayer. The organic thinfilm is most preferably a self-assembled
2 monolayer which comprises molecules of the formula X-R-Y, wherein R is a spacer, X is a
3 functional group that binds R to the surface, and Y is a functional group for binding protein-
4 capture agents onto the monolayer. In an alternative preferred embodiment, the self-
5 assembled monolayer is comprised of molecules of the formula $(X)_aR(Y)_b$ where a and b are,
6 independently, integers greater than or equal to 1 and X, R, and Y are as previously defined.
7 In an alternative preferred embodiment, the organic thinfilm comprises a combination of
8 organic thinfilms such as a combination of a lipid bilayer immobilized on top of a self-
9 assembled monolayer of molecules of the formula X-R-Y. As another example, a monolayer
10 of polylysine can also optionally be combined with a self-assembled monolayer of molecules
11 of the formula X-R-Y (see US Patent No. 5,629,213).

12 In all cases, the coating, or the substrate itself if no coating is present, must be
13 compatible with the chemical or physical adsorption of the organic thinfilm on its surface.
14 For instance, if the patches comprise a coating between the substrate and a monolayer of
15 molecules of the formula X-R-Y, then it is understood that the coating must be composed of
16 a material for which a suitable functional group X is available (see below). If no such
17 coating is present, then it is understood that the substrate must be composed of a material for
18 which a suitable functional group X is available.

19 In a preferred embodiment of the invention, the regions of the substrate surface, or
20 coating surface, which separate the patches of protein-capture agents are free of organic
21 thinfilm. In an alternative embodiment, the organic thinfilm extends beyond the area of the
22 substrate surface, or coating surface if present, covered by the patches of protein-capture

1 agents. For instance, optionally, the entire surface of the array may be covered by an organic
2 thinfilm on which the plurality of spatially distinct patches of protein-capture agents reside.
3 An organic thinfilm which covers the entire surface of the array may be homogenous or may
4 optionally comprise patches of differing exposed functionalities useful in the immobilization
5 of patches of different protein-capture agents. In still another alternative embodiment, the
6 regions of the substrate surface or coating surface, if a coating is present, between the
7 patches of protein-capture agents are covered by an organic thinfilm, but an organic thinfilm
8 of a different type than that of the patches of protein-capture agents. For instance, the
9 surfaces between the patches of protein-capture agents may be coated with an organic
10 thinfilm characterized by low non-specific binding properties for proteins and other analytes.

11 A variety of techniques may be used to generate patches of organic thinfilm on the
12 surface of the substrate or on the surface of a coating on the substrate. These techniques are
13 well known to those skilled in the art and will vary depending upon the nature of the organic
14 thinfilm, the substrate, and the coating if present. The techniques will also vary depending
15 on the structure of the underlying substrate and the pattern of any coating present on the
16 substrate. For instance, patches of a coating which is highly reactive with an organic
17 thinfilm may have already been produced on the substrate surface. Arrays of patches of
18 organic thinfilm can optionally be created by microfluidics printing, microstamping (US
19 Patent Nos. 5,512,131 and 5,731,152), or microcontact printing (μ CP) (PCT Publication WO
20 96/29629). Subsequent immobilization of protein-capture agents to the reactive monolayer
21 patches results in two-dimensional arrays of the agents. Inkjet printer heads provide another
22 option for patterning monolayer X-R-Y molecules, or components thereof, or other organic

1 thinfilm components to nanometer or micrometer scale sites on the surface of the substrate
2 or coating (Lemmo *et al.*, *Anal Chem.*, 1997, **69**:543-551; US Patent Nos. 5,843,767 and
3 5,837,860). In some cases, commercially available arrayers based on capillary dispensing
4 (for instance, OmniGrid™ from Genemachines, inc, San Carlos, CA, and High-Throughput
5 Microarrayer from Intelligent Bio-Instruments, Cambridge, MA) may also be of use in
6 directing components of organic thinfilms to spatially distinct regions of the array.

7 Diffusion boundaries between the patches of protein-capture agents immobilized on
8 organic thinfilms such as self-assembled monolayers may be integrated as topographic
9 patterns (physical barriers) or surface functionalities with orthogonal wetting behavior
10 (chemical barriers). For instance, walls of substrate material or photoresist may be used to
11 separate some of the patches from some of the others or all of the patches from each other.
12 Alternatively, non-bioreactive organic thinfilms, such as monolayers, with different
13 wettability may be used to separate patches from one another.

14 In yet another aspect, the invention provides for border regions that separate and/or
15 surround immobilization regions/patches, such borders having a first and selectively
16 achievable second wettable state. This enables an array to be spotted with solutions that are
17 contained in the immobilization regions by a first wettable state, preferably a hydrophobic
18 border, which constrains the fluid wholly or partly within the immobilization region, and
19 such border being further convertible to a second wettable state which is less hydrophobic,
20 or more hydrophilic than the first wettable state such that the fluid, and/or another fluid is
21 not contained within such immobilization regions by such border regions when such fluid is
22 placed within that immobilization region. Figures 10a-10d depicts how a droplet of fluid

1 may be contained within an immobilization or reaction region by a hydrophobic border, and
2 then not contained by the conversion of such borders to a hydrophilic or second wettable
3 state. Array 300 has border regions 100 adjacent, and in this case, abutting, immobilization
4 or reaction region 200. Drop 400 is placed on immobilization or reaction region 200 and
5 contained therein by the first wettable state of border regions 100. Light source LS is
6 activated in panel 10b which begins the conversion of border regions 100 from the first
7 wettable state to a second wettable state thus causing droplet 400 to spread out from
8 immobilization or reaction region 200 onto border regions 100. Panels 10c and 10d depict
9 how continued exposure to light from light source LS may further cause spreading until such
10 point at the border regions 100 assume fully their second wettable state, in this case one
11 similar to immobilization or reaction region 200. Other embodiments include having the
12 first wettable state be more hydrophilic than the second wettable state and or that the first and
13 second wettable states are reversible to one another, or that the reaction is just in one
14 direction. Border regions may further comprise a convertible functional group which
15 converts from a first wettable state to a second wettable state upon activation, preferably
16 where the activation is in the form activating a photocleavable group or a photoreactive
17 group, or a photoisomerizable group, or a catalytic-polymerization group within the
18 convertible function group. Other embodiments provide for methods employing such
19 convertible border arrays for making arrays of biomolecules attached to the surface of the
20 arrays, or for carrying out reactions on the surfaces of such arrays where such reactions are
21 contained within reaction regions surrounded wholly or partly by convertible border regions.

1 In a preferred embodiment of the invention, each of the patches of protein-capture
2 agents comprises a self-assembled monolayer of molecules of the formula X-R-Y, as
3 previously defined, and the patches are separated from each other by surfaces free of the
4 monolayer.

5 Figure 1 shows the top view of one example of an array of patches reactive with
6 protein-capture agents. On the array, a number of patches 15 cover the surface of the
7 substrate 3.

8 Figure 2 shows a detailed cross section of a patch 15 of the array of Figure 1. This
9 view illustrates the use of a coating 5 on the substrate 3. An adhesion interlayer 6 is also
10 included in the patch. On top of the patch resides a self-assembled monolayer 7.

11 Figure 3 shows a cross section of one row of the patches 15 of the array of Figure 1.
12 This figure also shows the use of a cover 2 over the array. Use of the cover 2 creates an inlet
13 port 16 and an outlet port 17 for solutions to be passed over the array.

14 A variety of chemical moieties may function as monolayer molecules of the formula
15 X-R-Y in the array of the present invention. However, three major classes of monolayer
16 formation are preferably used to expose high densities of reactive omega-functionalities on
17 the patches of the array: (i) alkylsiloxane monolayers ("silanes") on hydroxylated and non-
18 hydroxylated surfaces (as taught in, for example, US Patent No. 5,405,766, PCT Publication
19 WO 96/38726, US Patent No. 5,412,087, and US Patent No. 5,688,642); (ii) alkyl-
20 thiol/dialkyldisulfide monolayers on noble metals (preferably Au(111)) (as, for example,
21 described in Allara *et al.*, US 4,690,715; Bamdad *et al.*, US 5,620,850; Wagner *et al.*,
22 *Biophysical Journal*, 1996, 70:2052-2066); and (iii) alkyl monolayer formation on oxide-

1 free passivated silicon (as taught in, for example, Linford *et al.*, *J. Am. Chem. Soc.*, **1995**,
2 117:3145-3155, Wagner *et al.*, *Journal of Structural Biology*, 1997, **119**:189-201, US Patent
3 No. 5,429,708). One of ordinary skill in the art, however, will recognize that many possible
4 moieties may be substituted for X, R, and/or Y, dependent primarily upon the choice of
5 substrate, coating, and affinity tag. Many examples of monolayers are described in Ulman,
6 *An Introduction to Ultrathin Organic Films: From Langmuir-Blodgett to Self Assembly*,
7 Academic press (1991).

8 In one embodiment, the monolayer comprises molecules of the formula $(X)_aR(Y)_b$
9 wherein a and b are, independently, equal to an integer between 1 and about 200. In a
10 preferred embodiment, a and b are, independently, equal to an integer between 1 and about
11 80. In a more preferred embodiment, a and b are, independently, equal to 1 or 2. In a most
12 preferred embodiment, a and b are both equal to 1 (molecules of the formula X-R-Y).

13 If the patches of the invention array comprise a self-assembled monolayer of
14 molecules of the formula $(X)_aR(Y)_b$, then R may optionally comprise a linear or branched
15 hydrocarbon chain from about 1 to about 400 carbons long. The hydrocarbon chain may
16 comprise an alkyl, aryl, alkenyl, alkynyl, cycloalkyl, alkaryl, aralkyl group, or any
17 combination thereof. If a and b are both equal to one, then R is typically an alkyl chain from
18 about 3 to about 30 carbons long. In a preferred embodiment, if a and b are both equal to
19 one, then R is an alkyl chain from about 8 to about 22 carbons long and is, optionally, a
20 straight alkane. However, it is also contemplated that in an alternative embodiment, R may
21 readily comprise a linear or branched hydrocarbon chain from about 2 to about 400 carbons
22 long and be interrupted by at least one hetero atom. The interrupting hetero groups can

1 include -O-, -CONH-, -CONHCO-, -NH-, -CSNH-, -CO-, -CS-, -S-, -SO-, -(OCH₂CH₂)_n-
2 (where n=1-20), -(CF₂)_n- (where n=1-22), and the like. Alternatively, one or more of the
3 hydrogen moieties of R can be substituted with deuterium. In alternative, less preferred,
4 embodiments, R may be more than about 400 carbons long.

5 X may be chosen as any group which affords chemisorption or physisorption of the
6 monolayer onto the surface of the substrate (or the coating, if present). When the substrate
7 or coating is a metal or metal alloy, X, at least prior to incorporation into the monolayer, can
8 in one embodiment be chosen to be an asymmetrical or symmetrical disulfide, sulfide,
9 diselenide, selenide, thiol, isonitrile, selenol, a trivalent phosphorus compound,
10 isothiocyanate, isocyanate, xanthanate, thiocarbamate, a phosphine, an amine, thio acid or a
11 dithio acid. This embodiment is especially preferred when a coating or substrate is used that
12 is a noble metal such as gold, silver, or platinum.

13 If the substrate of the array is a material such as silicon, silicon oxide, indium tin
14 oxide, magnesium oxide, alumina, quartz, glass, or silica, then the array of one embodiment
15 of the invention comprises an X that, prior to incorporation into said monolayer, is a
16 monohalosilane, dihalosilane, trihalosilane, trialkoxysilane, dialkoxysilane, or a
17 monoalkoxysilane. Among these silanes, trichlorosilane and trialkoxysilane are particularly
18 preferred.

19 In a preferred embodiment of the invention, the substrate is selected from the group
20 consisting of silicon, silicon dioxide, indium tin oxide, alumina, glass, and titania; and X,
21 prior to incorporation into said monolayer, is selected from the group consisting of a

1 monohalosilane, dihalosilane, trihalosilane, trichlorosilane, trialkoxysilane, dialkoxysilane,
2 monoalkoxysilane, carboxylic acids, and phosphates.

3 In another preferred embodiment of the invention, the substrate of the array is silicon
4 and X is an olefin.

5 In still another preferred embodiment of the invention, the coating (or the substrate if
6 no coating is present) is titania or tantalum oxide and X is a phosphate.

7 In other embodiments, the surface of the substrate (or coating thereon) is composed
8 of a material such as titanium oxide, tantalum oxide, indium tin oxide, magnesium oxide, or
9 alumina where X is a carboxylic acid or alkylphosphoric acid. Alternatively, if the surface
10 of the substrate (or coating thereon) of the array is copper, then X may optionally be a
11 hydroxamic acid.

12 If the substrate used in the invention is a polymer, then in many cases a coating on
13 the substrate such as a copper coating will be included in the array. An appropriate
14 functional group X for the coating would then be chosen for use in the array. In an
15 alternative embodiment comprising a polymer substrate, the surface of the polymer may be
16 plasma-modified to expose desirable surface functionalities for monolayer formation. For
17 instance, EP 780423 describes the use of a monolayer molecule that has an alkene X
18 functionality on a plasma exposed surface. Still another possibility for the invention array
19 comprised of a polymer is that the surface of the polymer on which the monolayer is formed
20 is functionalized by copolymerization of appropriately functionalized precursor molecules.

21 Another possibility is that prior to incorporation into the monolayer, X can be a free-
22 radical-producing moiety. This functional group is especially appropriate when the surface

1 on which the monolayer is formed is a hydrogenated silicon surface. Possible free-radical
2 producing moieties include, but are not limited to, diacylperoxides, peroxides, and azo
3 compounds. Alternatively, unsaturated moieties such as unsubstituted alkenes, alkynes,
4 cyano compounds and isonitrile compounds can be used for X, if the reaction with X is
5 accompanied by ultraviolet, infrared, visible, or microwave radiation.

6 In alternative embodiments, X, prior to incorporation into the monolayer, may be a
7 hydroxyl, carboxyl, vinyl, sulfonyl, phosphoryl, silicon hydride, or an amino group.

8 The component, Y, of the monolayer is a functional group responsible for binding a
9 protein-capture agent onto the monolayer. In a preferred embodiment of the invention, the Y
10 group is either highly reactive (activated) towards the protein-capture agent (or its affinity
11 tag) or is easily converted into such an activated form. In a preferred embodiment, the
12 coupling of Y with the protein-capture agent occurs readily under normal physiological
13 conditions not detrimental to the ability of the protein-capture agent to bind its binding
14 partner. The functional group Y may either form a covalent linkage or a noncovalent linkage
15 with the protein-capture agent (or its affinity tag, if present). In a preferred embodiment, the
16 functional group Y forms a covalent linkage with the protein-capture agent or its affinity tag.
17 It is understood that following the attachment of the protein-capture agent (with or without
18 an affinity tag) to Y, the chemical nature of Y may have changed. Upon attachment of the
19 protein-capture agent, Y may even have been removed from the organic thinfilm.

20 In one embodiment of the array of the present invention, Y is a functional group that
21 is activated in situ. Possibilities for this type of functional group include, but are not limited
22 to, such simple moieties such as a hydroxyl, carboxyl, amino, aldehyde, carbonyl, methyl,

1 methylene, alkene, alkyne, carbonate, aryl iodide, or a vinyl group. Appropriate modes of
2 activation would be obvious to one skilled in the art. Alternatively, Y can comprise a
3 functional group that requires photoactivation prior to becoming activated enough to trap the
4 protein-capture agent.

5 In an especially preferred embodiment of the array of the present invention, Y is a
6 complex and highly reactive functional moiety that is compatible with monolayer formation
7 and needs no *in situ* activation prior to reaction with the protein-capture agent and/or affinity
8 tag. Such possibilities for Y include, but are not limited to, maleimide, N-
9 hydroxysuccinimide (Wagner *et al.*, *Biophysical Journal*, 1996, 70:2052-2066),
10 nitrilotriacetic acid (US Patent No. 5,620,850), activated hydroxyl, haloacetyl, bromoacetyl,
11 iodoacetyl, activated carboxyl, hydrazide, epoxy, aziridine, sulfonylchloride,
12 trifluoromethyldiaziridine, pyridyldisulfide, N-acyl-imidazole, imidazolecarbamate,
13 vinylsulfone, succinimidylcarbonate, arylazide, anhydride, diazoacetate, benzophenone,
14 isothiocyanate, isocyanate, imidoester, fluorobenzene, and biotin.

15 Figure 4 shows one example of a monolayer on a substrate 3. In this example,
16 substrate 3 comprises glass. The monolayer is thiolreactive because it bears a maleimidyl
17 functional group Y.

18 Figure 5 shows another example of a monolayer on a substrate 3 which is silicon. In
19 this case, however, a thin film gold coating 5 covers the surface of the substrate 3. Also, in
20 this embodiment, a titanium adhesion interlayer 6 is used to adhere the coating 5 to the
21 substrate 3. This monolayer is aminoreactive because it bears an N-hydroxysuccinimidyl
22 functional group Y.

1 In an alternative embodiment, the functional group Y of the array is selected from the
2 group of simple functional moieties. Possible Y functional groups include, but are not
3 limited to, -OH, -NH₂, -COOH, -COOR, -RSR, -PO₄⁻³, -OSO₃⁻², -SO₃⁻, -COO⁻, -SOO⁻, -
4 CONR₂, -CN, -NR₂, and the like.

5 The monolayer molecules of the present invention can optionally be assembled on
6 the surface in parts. In other words, the monolayer need not necessarily be constructed by
7 chemisorption or physisorption of molecules of the formula X-R-Y to the surface of the
8 substrate (or coating). Instead, in one embodiment, X may be chemisorbed or physisorbed to
9 the surface of the substrate (or coating) alone first. Then, R or even just individual
10 components of R can be attached to X through a suitable chemical reaction. Upon
11 completion of addition of the spacer R to the X moiety already immobilized on the surface,
12 Y can be attached to the ends of the monolayer molecule through a suitable covalent linkage.

13 Not all self-assembled monolayer molecules on a given patch need be identical to
14 one another. Some patches may comprise mixed monolayers. For instance, the monolayer
15 of an individual patch may optionally comprise at least two different molecules of the
16 formula X-R-Y, as previously described. This second X-R-Y molecule may immobilize the
17 same or a different protein-capture agent having the same binding partner as the first. In
18 addition, some of the monolayer molecules X-R-Y of a patch may have failed to attach any
19 protein-capture agent.

20 As another alternative embodiment of the invention, a mixed, self-assembled
21 monolayer of an individual patch on the array may comprise both molecules of the formula
22 X-R-Y, as previously described, and molecules of the formula, X-R-V where R is a spacer,

1 X is a functional group that binds R to the surface, and V is a moiety which is biocompatible
2 with proteins and resistant to the non-specific binding of proteins. For example, V may
3 consist of a hydroxyl, saccharide, or oligo/polyethylene glycol moiety (EP Publication
4 780423).

5 In still another embodiment of the invention, the array comprises at least one
6 unreactive patch of organic thinfilm on the substrate or coating surface which is devoid of
7 any protein-capture agent. For instance, the unreactive patch may optionally comprise a
8 monolayer of molecules of the formula X-R-V, where R is a spacer, X is a functional group
9 that binds R to the surface, and V is a moiety resistant to the non-specific binding of
10 proteins. The unreactive patch may serve as a control patch or be useful in background
11 binding measurements.

12 Regardless of the nature of the monolayer molecules, in some arrays it may be
13 desirable to provide crosslinking between molecules of an individual patch's monolayer. In
14 general, crosslinking confers additional stability to the monolayer. Such methods are
15 familiar to those skilled in the art (for instance, see Ulman, *An Introduction to Ultrathin*
16 *Organic Films: From Langmuir-Blodgett to Self-Assembly*, Academic Press (1991)).

17 After completion of formation of the monolayer on the patches, the protein-capture
18 agent may be attached to the monolayer via interaction with the Y-functional group. Y-
19 functional groups which fail to react with any protein-capture agents are preferably quenched
20 prior to use of the array.

21
22 (d) Affinity tags and immobilization of protein-capture agents.

1 In a preferred embodiment, the protein-immobilizing patches of the array further
2 comprise an affinity tag that enhances immobilization of the protein-capture agent onto the
3 organic thinfilm. The use of an affinity tag on the protein-capture agent of the array typically
4 provides several advantages. An affinity tag can confer enhanced binding or reaction of the
5 protein-capture agent with the functionalities on the organic thinfilm, such as Y if the
6 organic thinfilm is a an X-R-Y monolayer as previously described. This enhancement effect
7 may be either kinetic or thermodynamic. The affinity tag/thinfilm combination used in the
8 patches of the array preferably allows for immobilization of the protein-capture agents in a
9 manner which does not require harsh reaction conditions that are adverse to protein stability
10 or function. In most embodiments, immobilization to the organic thinfilm in aqueous,
11 biological buffers is ideal.

12 An affinity tag also preferably offers immobilization on the organic thinfilm that is
13 specific to a designated site or location on the protein-capture agent (site-specific
14 immobilization). For this to occur, attachment of the affinity tag to the protein-capture agent
15 must be site-specific. Site-specific immobilization helps ensure that the protein-binding site
16 of the agent, such as the antigen-binding site of the antibody moiety, remains accessible to
17 ligands in solution. Another advantage of immobilization through affinity tags is that it
18 allows for a common immobilization strategy to be used with multiple, different protein-
19 capture agents.

20 The affinity tag is optionally attached directly, either covalently or noncovalently, to
21 the protein-capture agent. In an alternative embodiment, however, the affinity tag is either

1 covalently or noncovalently attached to an adaptor which is either covalently or
2 noncovalently attached to the protein-capture agent.

3 In a preferred embodiment, the affinity tag comprises at least one amino acid. The
4 affinity tag may be a polypeptide comprising at least two amino acids which is reactive with
5 the functionalities of the organic thinfilm. Alternatively, the affinity tag may be a single
6 amino acid which is reactive with the organic thinfilm. Examples of possible amino acids
7 which could be reactive with an organic thinfilm include cysteine, lysine, histidine, arginine,
8 tyrosine, aspartic acid, glutamic acid, tryptophan, serine, threonine, and glutamine. A
9 polypeptide or amino acid affinity tag is preferably expressed as a fusion protein with the
10 protein-capture agent when the protein-capture agent is a protein, such as an antibody or
11 antibody fragment. Amino acid affinity tags provide either a single amino acid or a series of
12 amino acids that can interact with the functionality of the organic thinfilm, such as the Y-
13 functional group of the self-assembled monolayer molecules. Amino acid affinity tags can
14 be readily introduced into recombinant proteins to facilitate oriented immobilization by
15 covalent binding to the Y-functional group of a monolayer or to a functional group on an
16 alternative organic thinfilm.

17 The affinity tag may optionally comprise a poly(amino acid) tag. A poly(amino acid)
18 tag is a polypeptide that comprises from about 2 to about 100 residues of a single amino
19 acid, optionally interrupted by residues of other amino acids. For instance, the affinity tag
20 may comprise a poly-cysteine, polylysine, poly-arginine, or poly-histidine. Amino acid tags
21 are preferably composed of two to twenty residues of a single amino acid, such as, for
22 example, histidines, lysines, arginines, cysteines, glutamines, tyrosines, or any combination

1 of these. According to a preferred embodiment, an amino acid tag of one to twenty amino
2 acids includes at least one to ten cysteines for thioether linkage; or one to ten lysines for
3 amide linkage; or one to ten arginines for coupling to vicinal dicarbonyl groups. One of
4 ordinary skill in the art can readily pair suitable affinity tags with a given functionality on an
5 organic thinfilm.

6 The position of the amino acid tag can be at an amino-, or carboxy-terminus of the
7 protein-capture agent which is a protein, or anywhere in-between, as long as the protein-
8 binding region of the protein-capture agent, such as the antigen-binding region of an
9 immobilized antibody moiety, remains in a position accessible for protein binding. Where
10 compatible with the protein-capture agent chosen, affinity tags introduced for protein
11 purification are preferentially located at the C-terminus of the recombinant protein to ensure
12 that only full-length proteins are isolated during protein purification. For instance, if intact
13 antibodies are used on the arrays, then the attachment point of the affinity tag on the
14 antibody is preferably located at a C-terminus of the effector (Fc) region of the antibody. If
15 scFvs are used on the arrays, then the attachment point of the affinity tag is also preferably
16 located at the C-terminus of the molecules.

17 Affinity tags may also contain one or more unnatural amino acids. Unnatural amino
18 acids can be introduced using suppressor tRNAs that recognize stop codons (*i.e.*, amber)
19 (Noren *et al.*, *Science*, 1989, **244**:182-188; Ellman *et al.*, *Methods Enzym.*, 1991, **202**:301-
20 336; Cload *et al.*, *Chem. Biol.*, 1996, **3**:1033-1038). The tRNAs are chemically amino-
21 acylated to contain chemically altered (“unnatural”) amino acids for use with specific
22 coupling chemistries (*i.e.*, ketone modifications, photoreactive groups).

1 In an alternative embodiment the affinity tag can comprise an intact protein, such as,
2 but not limited to, glutathione S-transferase, an antibody, avidin, or streptavidin.

3 When the protein-capture agent is a protein and the affinity tag is a protein, such as a
4 poly(amino acid) tag, or a single amino acid, the affinity tag is preferably attached to the
5 protein-capture agent by generating a fusion protein. Alternatively, protein synthesis or
6 protein ligation techniques known to those skilled in the art may be used. For instance,
7 intein-mediated protein ligation may optionally be used to attach the affinity tag to the
8 protein-capture agent (Mathys, *et al.*, *Gene* **231**:1-13, 1999; Evans, *et al.*, *Protein Science*
9 **7**:2256-2264, 1998).

10 Other protein conjugation and immobilization techniques known in the art may be
11 adapted for the purpose of attaching affinity tags to the protein-capture agent. For instance,
12 in an alternative embodiment of the array, the affinity tag may be an organic bioconjugate
13 which is chemically coupled to the protein-capture agent of interest. Biotin or antigens may
14 be chemically cross linked to the protein. Alternatively, a chemical crosslinker may be used
15 that attaches a simple functional moiety such as a thiol or an amine to the surface of a
16 protein serving as a protein-capture agent on the array.

17 In an alternative embodiment of the invention, the organic thinfilm of each of the
18 patches comprises, at least in part, a lipid monolayer or bilayer, and the affinity tag
19 comprises a membrane anchor.

20 Figure 6 shows a detailed cross section of a patch on one embodiment of the
21 invention array. In this embodiment, a protein-capture agent 10 is immobilized on a
22 monolayer 7 on a substrate 3. An affinity tag 8 connects the protein-capture agent 10 to the

1 monolayer 7. The monolayer 7 is formed on a coating 5 which is separated from the
2 substrate 3 by an interlayer 6.

3 In an alternative embodiment of the invention, no affinity tag is used to immobilize
4 the protein-capture agents onto the organic thinfilm. An amino acid or other moiety (such as
5 a carbohydrate moiety) inherent to the protein-capture agent itself may instead be used to
6 tether the protein-capture agent to the reactive group of the organic thinfilm. In preferred
7 embodiments, the immobilization is site-specific with respect to the location of the site of
8 immobilization on the protein-capture agent. For instance, the sulfhydryl group on the C-
9 terminal region of the heavy chain portion of a Fab' fragment generated by pepsin digestion
10 of an antibody, followed by selective reduction of the disulfide between monovalent Fab'
11 fragments, may be used as the affinity tag. Alternatively, a carbohydrate moiety on the Fc
12 portion of an intact antibody can be oxidized under mild conditions to an aldehyde group
13 suitable for immobilizing the antibody on a monolayer via reaction with a hydrazide-
14 activated Y group on the monolayer. Examples of immobilization of protein-capture agents
15 without any affinity tag in a site-specific manner can be found in Dammer *et al.*, *Biophys J.*,
16 70:2437-2441, 1996 and the specific examples, Examples 5-7, below.

17 Since the protein-capture agents of at least some of the different patches on the array
18 are different from each other, different solutions, each containing a different, preferably,
19 affinity-tagged protein-capture agent, must be delivered to their individual patches.
20 Solutions of protein-capture agents may be transferred to the appropriate patches via arrayers
21 which are well-known in the art and even commercially available. For instance,
22 microcapillary-based dispensing systems may be used. These dispensing systems are

1 preferably automated and computer-aided. A description of and building instructions for an
2 example of a microarrayer comprising an automated capillary system can be found on the
3 internet at <http://cmgm.stanford.edu/pbrown/array.html> and
4 <http://cmgm.stanford.edu/pbrown/mguide/index.html>. The use of other microprinting
5 techniques for transferring solutions containing the protein-capture agents to the agent-
6 reactive patches is also possible. Ink-jet printer heads may also optionally be used for
7 precise delivery of the protein-capture agents to the agent-reactive patches. Representative,
8 non-limiting disclosures of techniques useful for depositing the protein-capture agents on the
9 patches may be found, for example, in U.S. Patent Nos. 5,731,152 (stamping apparatus),
10 5,807,522 (capillary dispensing device), 5,837,860 (ink-jet printing technique, Hamilton
11 2200 robotic pipetting delivery system), and 5,843,767 (ink-jet printing technique, Hamilton
12 2200 robotic pipetting delivery system), all incorporated by reference herein.

13
14 (e) Adaptors.

15 Another embodiment of the array of the present invention comprises an adaptor that
16 links the affinity tag to the protein-capture agent on the patches of the array. The additional
17 spacing of the protein-capture agent from the surface of the substrate (or coating) that is
18 afforded by the use of an adaptor is particularly advantageous if the protein-capture agent is
19 a protein, since proteins are known to be prone to surface inactivation. The adaptor may
20 optionally afford some additional advantages as well. For instance, the adaptor may help
21 facilitate the attachment of the protein-capture agent to the affinity tag. In another
22 embodiment, the adaptor may help facilitate the use of a particular detection technique with

1 the array. One of ordinary skill in the art will be able to choose an adaptor which is
2 appropriate for a given affinity tag. For instance, if the affinity tag is streptavidin, then the
3 adaptor could be biotin that is chemically conjugated to the protein-capture agent which is to
4 be immobilized.

5 In one embodiment, the adaptor comprises a protein. In another embodiment, the
6 affinity tag, adaptor, and protein-capture agent together compose a fusion protein. Such a
7 fusion protein may be readily expressed using standard recombinant DNA technology.

8 Adaptors which are proteins are especially useful to increase the solubility of the protein-
9 capture agent of interest and to increase the distance between the surface of the substrate or
10 coating and the protein-capture agent. Use of a protein adaptor can also be very useful in
11 facilitating the preparative steps of protein purification by affinity binding prior to
12 immobilization on the array. Examples of possible adaptor proteins include glutathione-S-
13 transferase (GST), maltose-binding protein, chitin-binding protein, thioredoxin, green-
14 fluorescent protein (GFP). GFP can also be used for quantification of surface binding. In a
15 preferred embodiment, when the protein-capture agent is an antibody moiety comprising the
16 Fc region, the adaptor is a polypeptide, such as protein G, protein A, or recombinant protein
17 A/G (a gene fusion product secreted from a non-pathogenic form of *Bacillus* which contains
18 four Fc binding domains from protein A and two from protein G).

19 Figure 7 shows a cross section of a patch on one particular embodiment of the
20 invention array. The patch comprises a protein-capture agent 10 immobilized on a
21 monolayer 7 via both an affinity tag 8 and an adaptor 9. The monolayer 7 rests on a coating
22 5. An interlayer 6 is used between the coating 5 and the substrate 3.

1
2 (f) Preparation of the protein-capture agents of the array.

3 The protein-capture agents used on the array may be produced by any of the variety
4 of means known to those of ordinary skill in the art. In a preferred embodiment of the
5 invention, the protein-capture agents are proteins, and in an especially preferred
6 embodiment, the protein-capture agents are antibodies or antibody fragments. Therefore,
7 methods of preparing these types of possible protein-capture agents are emphasized here.

8 In preparation for immobilization to the arrays of the present invention, the antibody
9 moiety, or any other protein-capture agent which is a protein or polypeptide, can optionally
10 be expressed from recombinant DNA either *in vivo* or *in vitro*. The cDNA of the antibody or
11 antibody fragment or other protein-capture agent is cloned into an expression vector (many
12 examples of which are commercially available) and introduced into cells of the appropriate
13 organism for expression. A broad range of host cells and expression systems may be used to
14 produce the antibodies and antibody fragments, or other proteins, which serve as the protein-
15 capture agents on the array. Expression *in vivo* may be done in bacteria (for example,
16 *Escherichia coli*), plants (for example, *Nicotiana tabacum*), lower eukaryotes (for example,
17 *Saccharomyces cerevisiae*, *Saccharomyces pombe*, *Pichia pastoris*), or higher eukaryotes
18 (for example, baculovirus-infected insect cells, insect cells, mammalian cells). For *in vitro*
19 expression PCR-amplified DNA sequences are directly used in coupled *in vitro*
20 transcription/translation systems (for instance: *Escherichia coli* S30 lysates from T7 RNA
21 polymerase expressing, preferably protease-deficient strains; wheat germ lysates;
22 reticulocyte lysates (Promega, Pharmacia, Panvera)). The choice of organism for optimal

1 expression depends on the extent of post-translational modifications (*i.e.*, glycosylation,
2 lipid-modifications) desired. The choice of expression system also depends on other issues,
3 such as whether an intact antibody is to be produced or just a fragment of an antibody (and
4 which fragment), since disulfide bond formation will be affected by the choice of a host cell.
5 One of ordinary skill in the art will be able to readily choose which host cell type is most
6 suitable for the protein-capture agent and application desired.

7 DNA sequences encoding affinity tags and adaptors can be engineered into the
8 expression vectors such that the protein-capture agent genes of interest can be cloned in
9 frame either 5' or 3' of the DNA sequence encoding the affinity tag and adaptor protein.

10 The expressed protein-capture agents are purified by affinity chromatography using
11 commercially available resins.

12 Preferably, production of a plurality of protein-capture agents involves parallel
13 processing from cloning to protein expression and protein purification. cDNAs for the
14 protein-capture agent of interest will be amplified by PCR using cDNA libraries or
15 expressed sequence tags (EST) clones as templates. For *in vivo* expression of the proteins,
16 cDNAs can be cloned into commercial expression vectors (Qiagen, Novagen, Clontech) and
17 introduced into an appropriate organism for expression (see above). For *in vitro* expression
18 PCR-amplified DNA sequences are directly used in coupled *in vitro* transcription/translation
19 systems (see above).

20 *Escherichia coli*-based protein expression is generally the method of choice for
21 soluble proteins that do not require extensive post-translational modifications for activity.

1 Extracellular or intracellular domains of membrane proteins will be fused to protein
2 adaptors for expression and purification.

3 The entire approach can be performed using 96-well assay plates. PCR reactions are
4 carried out under standard conditions. Oligonucleotide primers contain unique restriction
5 sites for facile cloning into the expression vectors. Alternatively, the TA cloning system
6 (Clontech) can be used. The expression vectors contain the sequences for affinity tags and
7 the protein adaptors. PCR products are ligated into the expression vectors (under inducible
8 promoters) and introduced into the appropriate competent *Escherichia coli* strain by
9 calcium-dependent transformation (strains include: XL-1 blue, BL21, SG13009(lon-)).
10 Transformed *Escherichia coli* cells are plated and individual colonies transferred into 96-
11 array blocks. Cultures are grown to mid-log phase, induced for expression, and cells
12 collected by centrifugation. Cells are resuspended containing lysozyme and the membranes
13 broken by rapid freeze/thaw cycles, or by sonication. Cell debris is removed by
14 centrifugation and the supernatants transferred to 96-tube arrays. The appropriate affinity
15 matrix is added, the protein-capture agent of interest is bound and nonspecifically bound
16 proteins are removed by repeated washing steps using 12 - 96 pin suction devices and
17 centrifugation. Alternatively, magnetic affinity beads and filtration devices can be used
18 (Qiagen). The proteins are eluted and transferred to a new 96-well array. Protein
19 concentrations are determined and an aliquot of each protein-capture agent is spotted onto a
20 nitrocellulose filter and verified by Western analysis using an antibody directed against the
21 affinity tag on the protein-capture agent. The purity of each sample is assessed by SDS-

PAGE and Silver staining or mass spectrometry. The protein-capture agents are then snap-frozen and stored at -80°C.

Saccharomyces cerevisiae allows for the production of glycosylated protein-capture agents such as antibodies or antibody fragments. For production in *Saccharomyces cerevisiae*, the approach described above for *Escherichia coli* can be used with slight modifications for transformation and cell lysis. Transformation of *Saccharomyces cerevisiae* is by lithium-acetate and cell lysis is either by lyticase digestion of the cell walls followed by freeze-thaw, sonication or glass-bead extraction. Variations of post-translational modifications can be obtained by using different yeast strains (*i.e.*, *Saccharomyces pombe*, *Pichia pastoris*).

One aspect of the baculovirus system is the array of post-translational modifications that can be obtained, although antibodies and other proteins produced in baculovirus contain carbohydrate structures very different from those produced by mammalian cells. The baculovirus-infected insect cell system requires cloning of viruses, obtaining high titer stocks and infection of liquid insect cell suspensions (cells such as SF9, SF21).

Mammalian cell-based expression requires transfection and cloning of cell lines. Either lymphoid or non-lymphoid cell may be used in the preparation of antibodies and antibody fragments. Soluble proteins such as antibodies are collected from the medium while intracellular or membrane bound proteins require cell lysis (either detergent solubilization, freeze-thaw). The protein-capture agents can then be purified analogous to the procedure described for *Escherichia coli*.

1 For *in vitro* translation the system of choice is *Escherichia coli* lysates obtained from
2 protease-deficient and T7 RNA polymerase overexpressing strains. *Escherichia coli* lysates
3 provide efficient protein expression (30-50 µg/ml lysate). The entire process is carried out
4 in 96-well arrays. Antibody genes or other protein-capture agent genes of interest are
5 amplified by PCR using oligonucleotides that contain the gene-specific sequences containing
6 a T7 RNA polymerase promoter and binding site and a sequence encoding the affinity tag.
7 Alternatively, an adaptor protein can be fused to the gene of interest by PCR. Amplified
8 DNAs can be directly transcribed and translated in the *Escherichia coli* lysates without prior
9 cloning for fast analysis. The antibody fragments or other proteins are then isolated by
10 binding to an affinity matrix and processed as described above.

11 Alternative *in vitro* translation systems which may be used include wheat germ
12 extracts and reticulocyte extracts. *In vitro* synthesis of membrane proteins or post-
13 translationally modified proteins will require reticulocyte lysates in combination with
14 microsomes.

15 In one embodiment of the invention, the protein-capture agents on the array are
16 monoclonal antibodies. The production of monoclonal antibodies against specific protein
17 targets is routine using standard hybridoma technology. In fact, numerous monoclonal
18 antibodies are available commercially. The preparation and use of an array of monoclonal
19 antibodies is illustrated in the specific example, Example 8, below.

20 As an alternative to obtaining antibodies or antibody fragments by cell fusion or from
21 continuous cell lines, the antibody moieties may be expressed in bacteriophage. Such
22 antibody phage display technologies are well known to those skilled in the art. The

1 bacteriophage expression systems allow for the random recombination of heavy- and light-
2 chain sequences, thereby creating a library of antibody sequences which can be selected
3 against the desired antigen. The expression system can be based on bacteriophage λ or ,
4 more preferably, on filamentous phage. The bacteriophage expression system can be used to
5 express Fab fragments, Fv's with an engineered intermolecular disulfide bond to stabilize
6 the V_H - V_L pair (dsFv's), scFvs, or diabody fragments.

7 The antibody genes of the phage display libraries may be from pre-immunized
8 donors. For instance, the phage display library could be a display library prepared from the
9 spleens of mice previously immunized with a mixture of proteins (such as a lysate of human
10 T-cells). Immunization can optionally be used to bias the library to contain a greater number
11 of recombinant antibodies reactive towards a specific set of proteins (such as proteins found
12 in human T-cells). Alternatively, the library antibodies may be derived from naive or
13 synthetic libraries. The naive libraries have been constructed from spleens of mice which
14 have not been contacted by external antigen. In a synthetic library, portions of the antibody
15 sequence, typically those regions corresponding to the complementarity determining regions
16 (CDR) loops, have been mutagenized or randomized.

17 The phage display method involves batch-cloning the antibody gene library into a
18 phage genome as a fusion to the gene encoding one of the phage coat proteins (pIII, pVI, or
19 pVIII). The pIII phage protein gene is preferred. When the fusion product is expressed it is
20 incorporated into the mature phage coat. As a result, the antibody is displayed as a fusion on
21 the surface of the phage and is available for binding and hence, selection, on a target protein.
22 Once a phage particle is selected as bearing an antibody-coat protein fusion with the desired

1 affinity towards the target protein, the genetic material within the phage particle which
2 corresponds to the displayed antibody can be amplified and sequenced or otherwise
3 analyzed.

4 In a preferred embodiment, a phagemid is used as the expression vector in the
5 phage display procedures. A phagemid is a small plasmid vector that carries gene III with
6 appropriate cloning sites and a phage packaging signal and contains both host and phage
7 origins of replication. The phagemid is unable to produce a complete phage as the gene III
8 fusion is the only phage gene encoded on the phagemid. A viable phage can be produced by
9 infecting cells containing the phagemid with a helper phage containing a defective
10 replication origin. A hybrid phage emerges which contains all of the helper phage proteins
11 as well as the gene III-rAb fusion. The emergent phage contains the phagemid DNA only.

12 In a preferred embodiment of the invention, the recombinant antibodies used in
13 phage display methods of preparing protein-capture agents for the arrays of the invention are
14 expressed as genetic fusions to the bacteriophage gene III protein on a phagemid vector. For
15 instance, the antibody variable regions encoding a single-chain Fv fragment can be fused to
16 the amino terminus of the gene III protein on a phagemid. Alternatively, the antibody
17 fragment sequence could be fused to the amino terminus of a truncated pIII sequence lacking
18 the first two N-terminal domains. The phagemid DNA encoding the antibody-pIII fusion is
19 preferably packaged into phage particles using a helper phage such as M13KO7 or VCS-
20 M13, which supplies all structural phage proteins.

1 To display Fab fragments on phage, either the light or heavy (Fd) chain is fused via
2 its C-terminus to pIII. The partner chain is expressed without any fusion to pIII so that both
3 chains can associate to form an intact Fab fragment.

4 Any method of selection may be used which separates those phage particles which do
5 bind the target protein from those which do not. The selection method must also allow for
6 the recovery of the selected phages. Most typically, the phage particles are selected on an
7 immobilized target protein. Some phage selection strategies known to those skilled in the art
8 include the following: panning on an immobilized antigen; panning on an immobilized
9 antigen using specific elution; using biotinylated antigen and then selecting on a streptavidin
10 resin or streptavidin-coated magnetic beads; affinity purification; selection on Western blots
11 (especially useful for unknown antigens or antigens difficult to purify); *in vivo* selection; and
12 pathfinder selection. If the selected phage particles are amplified between selection rounds,
13 multiple iterative rounds of selection may optionally be performed.

14 Elution techniques will vary depending upon the selection process chosen, but typical
15 elution techniques include washing with one of the following solutions: HCl or glycine
16 buffers; basic solutions such as triethylamine; chaotropic agents; solutions of increased ionic
17 strength; or DTT when biotin is linked to the antigen by a disulfide bridge. Other typical
18 methods of elution include enzymatically cleaving a protease site engineered between the
19 antibody and gene III, or by competing for binding with excess antigen or excess antibodies
20 to the antigen.

21 A method for producing an array of antibody fragments therefore comprises first
22 selecting recombinant bacteriophage which express antibody fragments from a phage display

1 library. The recombinant bacteriophage are selected by affinity binding to a protein which is
2 an expression product, or fragment thereof, of a cell or population of cells in an organism.
3 (Iterative rounds of selection are possible, but optional.) Next, at least one purified sample
4 of an antibody fragment from a bacteriophage which was selected in the first step is
5 produced. This antibody production step typically entails infecting *E. coli* cells with the
6 selected bacteriophage. In the absence of helper phage, the selected bacteriophage then
7 replicate as expressive plasmids without producing phage progeny. Alternatively, the
8 antibody fragment gene of the selected recombinant bacteriophage is isolated, amplified, and
9 then expressed in a suitable expression system. In either case, following amplification, the
10 expressed antibody fragment of the selected and amplified recombinant bacteriophage is
11 isolated and purified. In a third step of the method, the earlier steps of phage display
12 selection and purified antibody fragment production are repeated using affinity binding to
13 different proteins which are expression products, or fragments thereof, of the same cell or
14 population of cells as before until the desired plurality of purified samples of different
15 antibodies with different binding pairs are produced. In a final step of the method, the
16 antibody fragment of each different purified sample is immobilized onto an organic thinfilm
17 on a separate patch on the surface of a substrate to form a plurality of patches of antibody
18 fragments on discrete, known regions of the substrate surface covered by organic thinfilm.

19 For instance, to generate an antibody array with antibody fragments against known
20 protein targets, open reading frames of the known protein targets identified in DNA
21 databases are amplified by polymerase chain reaction and transcribed and translated *in vitro*
22 to produce proteins on which a recombinant bacteriophage expressing single-chain antibody

1 fragments are selected. Once selected, the antibody fragment sequence of the selected
2 bacteriophage is amplified (typically using the polymerase chain method) and recloned into a
3 desirable expression system. The expressed antibody fragments are purified and then printed
4 onto organic thinfilms on substrates to form the high density arrays.

5 In another embodiment of the invention, a method for producing an array of protein-
6 capture agents is provided which comprises first selecting protein-capture agents from a
7 library of protein-capture agents, where the protein-capture agents are selected by their
8 affinity binding to the proteins from a cellular extract or body fluid. Preferably, the proteins
9 are from a cellular extract. The proteins from the cellular extract or body fluid would
10 typically be immobilized prior to the selection step. Suitable methods of immobilization
11 such as crosslinking of the proteins to a resin are well known to one of ordinary skill in the
12 art. The next step of this method comprises producing a plurality of purified samples of the
13 selected protein-capture agents. The protein-capture agent of each different purified sample
14 is immobilized onto an organic thinfilm on a separate patch on the surface of a substrate to
15 form a plurality of patches of protein-capture agents on discrete, known regions of the
16 substrate surface covered by organic thinfilm.

17 This method of array preparation optionally also comprises the additional step of
18 biasing the library of protein-capture agents by eliminating from the library those protein-
19 capture agents which bind certain proteins, such as the proteins of a second cellular extract,
20 wherein the protein-capture agents which are eliminated are removed from the library by
21 their binding affinity to those certain proteins. This step of biasing the library may
22 optionally occur after the selection step by affinity binding to the protein, but more typically,

1 it occurs prior to that selection step. The order of the selecting and biasing steps will depend
2 on the nature of the selection and elution procedures used in the method. One of ordinary
3 skill in the art will readily be able to determine an appropriate series of steps.

4 In one embodiment of the optional step of biasing the library of protein-capture
5 agents, the library is biased to eliminate protein-capture agents that recognize common
6 proteins or proteins of non-interest. This is typically achieved by passing the library over an
7 affinity surface, such as a chromatography column, containing cross-linked proteins of non-
8 interest. The "flowthrough" containing protein-capture agents that did not react with the
9 affinity surface is collected. This procedure enriches the library for protein-capture agents
10 which bind proteins of interest or proteins specific to the cell to be assayed. For instance, if
11 the library is derived from a specific cell type such as a human T-cell, the library may
12 optionally be biased by passing it over an affinity surface which contains proteins prepared
13 from a lysate of human fibroblasts or bacterial proteins to enrich the library for protein-
14 capture agents which bind proteins specifically present in fibroblasts.

15 In a preferred embodiment of the method of preparing the array of protein-capture
16 agents described above, the protein-capture agents are antibody fragments displayed on the
17 surface of recombinant bacteriophages and the library of protein-capture agents is a phage
18 display library. Therefore, a method for producing an antibody array comprises first
19 selecting recombinant bacteriophage expressing antibody fragments from a phage display
20 library, where the bacteriophage are selected by affinity binding to immobilized proteins of a
21 body fluid, or more preferably, a cellular extract. The next step of this method comprises
22 producing a plurality of purified samples of antibody fragments expressed by the selected

1 recombinant bacteriophage. Preferably, antibody fragments which specifically bind more
2 than 1000 of the proteins of the cellular extract are produced in this manner. In a final step
3 of the method, the antibody fragment of each different purified sample is immobilized onto
4 an organic thinfilm on a separate patch on the surface of a substrate to form a plurality of
5 patches of antibody fragments on discrete known regions of the substrate surface. One
6 specific example of this method is outlined in Example 6, below. Again, this method
7 optionally also comprises the additional step of biasing the phage display library by
8 eliminating from the library those bacteriophage displaying antibody fragments which bind
9 certain proteins, such as the proteins of a second cellular extract. The bacteriophage which
10 are eliminated are removed from the library by the binding affinity of their displayed
11 antibody fragments to the certain proteins.

12 For instance, a method of preparing an antibody array optionally begins with
13 a phage display library prepared from RNA isolated from the spleens of mice previously
14 immunized with a lysate of human T-cells. The phage library is then passed over a column
15 or affinity surface comprising proteins from the lysates of background cells such as human
16 fibroblasts which have been cross-linked to a surface or resin. The phage remaining in the
17 flowthrough solution from the first column/affinity surface is then passed over a second
18 affinity surface, such as a chromatography column, containing cross-linked proteins prepared
19 from a lysate of human T-cells. The flowthrough solution from the second column/affinity
20 surface is then discarded since this solution contains phage which displays recombinant
21 antibodies that did not react with the second affinity surface. Phage which specifically react
22 with the second affinity surface and remain bound to the second affinity surface are then

1 collected by elution. Elution can be achieved by lowered pH (2.0), increased ionic strength,
2 or proteolytic release by a specific proteolytic cut site genetically engineered between the
3 displayed recombinant antibody and the gene III protein of the phage. In a next step of the
4 method, the eluted phage are separated into isolated plaques by plating and then propagated
5 as separate cultures. Periplasmic fractions from the separate cultures are prepared and the
6 corresponding recombinant antibodies purified. The purified recombinant antibodies are
7 then dispensed into separate patches on a 2-D array where they are immobilized onto an
8 organic thinfilm.

9 Methods of preparing an array of protein-capture agents where the protein-
10 capture agents have been selected against the proteins of a cellular extract, or a body fluid,
11 create arrays of protein-capture agents where all of the binding partners of the arrays are not
12 initially known. The primary information provided by binding of proteins to these types of
13 arrays is contained in the pattern of protein abundance. Once interesting patches on an array
14 have been identified by comparison of the protein expression pattern to that of a control (for
15 instance, it may be observed that there is a significant increase in the amount of protein
16 bound to a patch of the array following exposure of a cell to a certain set of conditions), the
17 identity of the protein ligand binding to a particular patch on the array can be assessed by
18 affinity purification of the protein ligand followed by microsequencing and/or mass
19 spectrometry or the like.

20 An alternative method for producing an array of protein-capture agents
21 comprises: selecting protein-capture agents from a library of protein-capture agents, wherein
22 the protein-capture agents are selected by their binding affinity to proteins expressed by a

1 cDNA expression library; producing a plurality of purified samples of the selected protein-
2 capture agents; and immobilizing each different purified protein-capture agent onto an
3 organic thinfilm on a separate patch on the surface of a substrate to form a plurality of
4 patches on discrete, known regions of the substrate surface covered by organic thinfilm

5 This method also optionally comprises the additional step of biasing the
6 protein-capture agent library by eliminating from the library those protein-capture agents
7 which bind certain proteins, such as the proteins of a cellular extract, wherein the protein-
8 capture agents which are eliminated are removed from the library by their binding affinity to
9 said certain proteins. In most cases, the proteins which are used to subtract protein-capture
10 agents from the library of protein-capture agents would be immobilized. This step of biasing
11 the library may optionally occur after the selection step by affinity binding to the proteins
12 expressed by the cDNA expression library, but more typically, it occurs prior to that
13 selection step. The order of these step will depend on the nature of the selection and elution
14 steps. One of ordinary skill in the art will readily be able to determine an appropriate series
15 of steps. In the optional step of biasing the library of protein-capture agents, the library is
16 optionally biased to eliminate protein-capture agents that recognize common proteins or
17 proteins of non-interest (as described above for a previous embodiment). Preferably, the
18 method further comprises the additional step of identifying which individual selected
19 protein-capture agents bind which individual proteins expressed by the cDNA expression
20 library.

1 In another preferred embodiment of the the method, the protein-capture
2 agents are antibody fragments displayed on the surface of recombinant bacteriophages and
3 the library of protein-capture agents is a phage display library.

4 For instance, one example of a method of preparing an array of antibodies
5 optionally begins with a phage display library prepared from RNA isolated from the spleens
6 of mice previously immunized with a lysate of human T-cells. The phage library is then
7 passed over a column or affinity surface comprising proteins from the lysates of background
8 cells such as human fibroblasts which have been cross-linked to a surface or resin. The
9 phage remaining in the flowthrough solution from the first column/affinity surface is then
10 collected. A cDNA expression library derived from message RNA (mRNA) isolated from
11 human T-cells is prepared in which the expressed proteins from the expression library are
12 genetically fused with an expression tag (such as a six histidine tag). The library is
13 expanded and the tagged proteins are collectively expressed and purified. The pool of
14 purified, tagged proteins from the cDNA expression library is cross-linked to an affinity
15 surface, such as a chromatography column. The phage display library which passed through
16 the first affinity surface or column is passed over the affinity surface bearing the
17 immobilized proteins of the cDNA expression library. The flowthrough solution containing
18 phage displaying recombinant antibodies that did not react with the affinity surface is
19 discarded. Phage which specifically react with the affinity surface are collected by elution
20 achieved by lowering the pH (2.0). Cells from the cDNA expression library are plated and a
21 filter lift of the colonies is made using nitrocellulose or charged nylon filters. Reactive sites
22 on the filter are blocked with a standard blocking solution and the filters are probed with the

1 selected bacteriophage eluted off of the second column. The phage are visualized by
2 reaction with a monoclonal antibody recognizing the gene VIII coat protein of the
3 bacteriophage, conjugated to alkaline phosphatase. Reactive sites on the filter are cut out and
4 the phage eluted from the filter pieces and propagated separately. The eluted phage are
5 separated into isolated plaques and then propagated as separate cultures. Periplasmic
6 fractions from the separate cultures are prepared and the corresponding recombinant
7 antibodies purified. The purified recombinant antibodies are then dispensed onto separate
8 patches of organic thinfilm on a 2-D array. Samples are reacted with the array and protein
9 ligands with interesting differential abundance patterns (when compared to a control) are
10 identified. Colonies on the original plate corresponding to the phage-reactive sites on the
11 filter are propagated and the plasmids containing the cDNA sequenced to identify the protein
12 ligands reactive with the recombinant antibodies of the phage.

13 In the preparation of the arrays of the invention, phage display methods analogous to
14 those used for antibody fragments may be used for protein-capture agents other than
15 antibody fragments as long as the protein-capture agent is composed of protein and is of
16 suitable size to be incorporated into the phagemid or alternative vector and expressed as a
17 fusion with a bacteriophage coat protein. Phage display techniques using non-antibody
18 libraries typically make use of some type of protein host scaffold structure which supports
19 the variable regions. For instance, β -sheet proteins, α -helical handle proteins, and other
20 highly constrained protein structures have been used as host scaffolds.

21 Alternative display vectors may also be used to produce the protein-capture agents,
22 such as antibody moieties, which are printed on the arrays of the invention. Polysomes,

1 stable protein-ribosome-mRNA complexes, can be used to replace live bacteriophage as the
2 display vehicle for recombinant antibody fragments or other proteins (Hanes and Pluckthun,
3 *Proc. Natl. Acad. Sci USA*, **94**:4937-4942, 1997). The polysomes are formed by preventing
4 release of newly synthesized and correctly folded protein from the ribosome. Selection of
5 the polysome library is based on binding of the antibody fragments or other proteins which
6 are displayed on the polysomes to the target protein. mRNA which encodes the displayed
7 protein or antibody having the desired affinity for the target is then isolated. Larger libraries
8 may be used with polysome display than with phage display.

9 In still another alternative method of preparing the protein-capture agents of the
10 arrays of the invention, an alternative display method of selection such as lambda display
11 (Mikawa *et al.*, *J. Mol. Biol.*, **262**:21-30, 1996), bacterial display (Georgiou *et al.*, *Nat.*
12 *Biotechnol.*, **15**:29-34, 1997) or eukaryotic cell display may instead be used.

13 Furthermore, selection methods other than display methods may also be used in the
14 preparation of protein-capture agents for the arrays of the invention. As indicated above, the
15 protein-capture agents may be obtained by any *in vitro* or *in vivo* selection procedure known
16 to those skilled in the art. In one embodiment of the invention, protein-capture agents other
17 than antibodies and antibody fragments are batch selected on the protein in cellular extracts.
18 Such procedures generate a diversity of protein-capture agents which are highly suitable for
19 applications in proteomics.

20 In alternative embodiments of the invention, the protein-capture agents are partially
21 or wholly prepared by synthetic means. If the protein-capture agent is a protein, then
22 methods of peptide synthetic or protein ligation may optionally be used to construct a protein

1 from amino acid or polypeptide building blocks. Protein-capture agents which are
2 polynucleotides are readily prepared synthetically.

3
4 (g) Uses of the arrays.

5 The present invention also provides methods of using the invention arrays. In
6 general, for a variety of applications including proteomics and diagnostics, the methods of
7 the invention involve the delivery of the sample containing the proteins to be analyzed to the
8 arrays. After the proteins of the sample have been allowed to interact with and become
9 immobilized on the patches of the array comprising protein-capture agents with the
10 appropriate biological specificity, the presence and/or amount of protein bound at each patch
11 is then determined.

12 Use of one of the protein-capture agent arrays of the invention may optionally
13 involve placing the two-dimensional array in a flowchamber with approximately 1-10
14 microliters of fluid volume per 25 mm² overall surface area. The cover over the array in the
15 flowchamber is preferably transparent or translucent. In one embodiment, the cover may
16 comprise Pyrex or quartz glass. In other embodiments, the cover may be part of a detection
17 system that monitors interaction between the protein-capture agents immobilized on the
18 array and protein in a solution such as a cellular extract. The flowchambers should remain
19 filled with appropriate aqueous solutions to preserve protein activity. Salt, temperature, and
20 other conditions are preferably kept similar to those of normal physiological conditions.
21 Proteins in a fluid solution may be flushed into the flow chamber as desired and their
22 interaction with the immobilized protein-capture agents determined. Sufficient time must be

1 given to allow for binding between the protein-capture agent and its binding partner to occur.
2 The amount of time required for this will vary depending upon the nature and tightness of
3 the affinity of the protein-capture agent for its binding partner. No specialized microfluidic
4 pumps, valves, or mixing techniques are required for fluid delivery to the array.

5 Alternatively, protein-containing fluid can be delivered to each of the patches of the
6 array individually. For instance, in one embodiment, the regions of the substrate surface
7 may be microfabricated in such a way as to allow integration of the array with a number of
8 fluid delivery channels oriented perpendicular to the array surface, each one of the delivery
9 channels terminating at the site of an individual protein-capture agent-coated patch.

10 The sample which is delivered to the array will typically be a fluid. In a preferred
11 embodiment of the invention, the sample is a cellular extract or a body fluid. The sample to
12 be assayed may optionally comprise a complex mixture of proteins, including a multitude of
13 proteins which are not binding partners of the protein-capture agents of the array. If the
14 proteins to be analyzed in the sample are membrane proteins, then those proteins will
15 typically need to be solubilized prior to administration of the sample to the array. If the
16 proteins to be assayed in the sample are proteins secreted by a population of cells in an
17 organism, a sample which is derived from a body fluid is preferred. If the proteins to be
18 assayed in the sample are intracellular, a sample which is a cellular extract is preferred. In
19 one embodiment of the invention, the array may comprise protein-capture agents which bind
20 fragments of the expression products of a cell or population of cells in an organism. In such
21 a case, the proteins in the sample to be assayed may have been prepared by performing a
22 digest of the protein in a cellular extract or a body fluid. In an alternative application of the

1 array, the proteins from only specific fractions of a cell are collected for analysis in the
2 sample.

3 In general, delivery of solutions containing proteins to be bound by the protein-
4 capture agents of the array may optionally be preceded, followed, or accompanied by
5 delivery of a blocking solution. A blocking solution contains protein or another moiety
6 which will adhere to sites of non-specific binding on the array. For instance, solutions of
7 bovine serum albumin or milk may be used as blocking solutions.

8 It is understood that some proteins a sample which are not the intended binding
9 partner of the protein-capture agents of a patch (and may, in fact, be the intended binding
10 partner of another patch) on the array may still bind to the patch to some degree.
11 Preferably, this type of binding only occurs to a very minor degree. Also, it is understood
12 that even when the correct binding partners are present in the solution being assayed, the
13 binding partners will bind to the patch comprising their protein-capture agent with less than
14 100% efficiency.

15 A wide range of detection methods is applicable to the methods of the invention. As
16 desired, detection may be either quantitative or qualitative. The invention array can be
17 interfaced with optical detection methods such as absorption in the visible or infrared range,
18 chemoluminescence, and fluorescence (including lifetime, polarization, fluorescence
19 correlation spectroscopy (FCS), and fluorescence-resonance energy transfer (FRET)).
20 Furthermore, other modes of detection such as those based on optical waveguides PCT
21 Publication (WO 96/26432 and U.S. Patent No. 5,677,196), surface plasmon resonance,
22 surface charge sensors, and surface force sensors are compatible with many embodiments of

1 the invention. Alternatively, technologies such as those based on Brewster Angle
2 microscopy (BAM) (Schaaf *et al.*, *Langmuir*, 3:1131-1135 (1987)) and ellipsometry (U.S.
3 Patent Nos. 5,141,311 and 5,116,121; Kim, *Macromolecules*, 22:2682-2685 (1984)) could
4 be applied. Quartz crystal microbalances and desorption processes (see for example, U.S.
5 Patent No. 5,719,060) provide still other alternative detection means suitable for at least
6 some embodiments of the invention array. An example of an optical biosensor system
7 compatible both with some arrays of the present invention and a variety of non-label
8 detection principles including surface plasmon resonance, total internal reflection
9 fluorescence (TIRF), Brewster Angle microscopy, optical waveguide lightmode
10 spectroscopy (OWLS), surface charge measurements, and ellipsometry can be found in U.S.
11 Patent No. 5,313,264.

12 Although non-label detection methods are generally preferred, some of the types of
13 detection methods commonly used for traditional immunoassays which require the use of
14 labels may be applied to the arrays of the present invention. These techniques include
15 noncompetitive immunoassays, competitive immunoassays, and dual label, ratiometric
16 immunoassays. These particular techniques are primarily suitable for use with the arrays of
17 protein-capture agents when the number of different protein-capture agents with different
18 specificity is small (less than about 100). In the competitive method, binding-site occupancy
19 is determined indirectly. In this method, the protein-capture agents of the array are exposed
20 to a labeled developing agent, which is typically a labeled version of the analyte or an
21 analyte analog. The developing agent competes for the binding sites on the protein-capture
22 agent with the analyte. The fractional occupancy of the protein-capture agents on different

1 patches can be determined by the binding of the developing agent to the protein-capture
2 agents of the individual patches. In the noncompetitive method, binding site occupancy is
3 determined directly. In this method, the patches of the array are exposed to a labeled
4 developing agent capable of binding to either the bound analyte or the occupied binding sites
5 on the protein-capture agent. For instance, the developing agent may be a labeled antibody
6 directed against occupied sites (*i.e.*, a “sandwich assay”). Alternatively, a dual label,
7 ratiometric, approach may be taken where the protein-capture agent is labeled with one label
8 and the second, developing agent is labeled with a second label (Ekins, *et al.*, *Clinica*
9 *Chimica Acta.*, **194**:91-114, 1990). Many different labeling methods may be used in the
10 aforementioned techniques, including radioisotopic, enzymatic, chemiluminescent, and
11 fluorescent methods. Fluorescent methods are preferred.

12 Figure 8 shows a schematic diagram of one type of fluorescence detection unit which
13 may be used to monitor interaction of immobilized protein-capture agents of an array with a
14 protein analyte. In the illustrated detection unit, the array of protein-capture agents 21 is
15 positioned on a base plate 20. Light from a 100W mercury arc lamp 25 is directed through
16 an excitation filter 24 and onto a beam splitter 23. The light is then directed through a lens
17 22, such as a Micro Nikkor 55 mm 1:2.8 lens, and onto the array 21. Fluorescence emission
18 from the array returns through the lens 22 and the beam splitter 23. After next passing
19 through an emission filter 26, the emission is received by a cooled CCD camera 27, such as
20 the Slowsan TE/CCD-1024SF&SB (Princeton Instruments). The camera is operably
21 connected to a CPU 28 which is in turn operably connected to a VCR 29 and a monitor 30.

1 Figure 9 shows a schematic diagram of an alternative detection method based on
2 ellipsometry. Ellipsometry allows for information about the sample to be determined from
3 the observed change in the polarization state of a reflected light wave. Interaction of a
4 protein analyte with a layer of immobilized protein-capture agents on a patch results in a
5 thickness change and alters the polarization status of a plane-polarized light beam reflected
6 off the surface. This process can be monitored *in situ* from aqueous phase and, if desired, in
7 imaging mode. In a typical setup, monochromatic light (e.g. from a He-Ne laser, 30) is
8 plane polarized (polarizer 31) and directed onto the surface of the sample and detected by a
9 detector 35. A compensator 32 changes the elliptically polarized reflected beam to plane-
10 polarized. The corresponding angle is determined by an analyzer 33 and then translated into
11 the ellipsometric parameters Psi and Delta which change upon binding of protein with the
12 protein-capture agents. Additional information can be found in Azzam, et al., *Ellipsometry*
13 *and Polarized Light*, North-Holland Publishing Company: Amsterdam, 1977.

14 The arrays of the present invention are particularly useful for proteomics. Those
15 arrays which comprise significant numbers of protein-capture agents of different specificity
16 on separate patches can bind significant numbers of proteins which are expression products,
17 or fragments thereof, of a cell or population of cells in an organism and are particularly
18 suitable for use in applications involving proteomics. For instance, an array with at least
19 about 10^3 and up to about 10^5 different protein-capture agents such as antibodies or antibody
20 fragments can provide a highly comprehensive picture of the protein content of the cell
21 under a specific set of conditions.

1 In one embodiment of the invention, a method of assaying in parallel for a plurality
2 of different proteins in a sample which are expression products, or fragments thereof, of a
3 cell or a population of cells in an organism, is provided which comprises the following steps:
4 first, delivering the sample to an array of spatially distinct patches of different protein-
5 capture agents under conditions suitable for protein binding, wherein each of the proteins
6 being assayed is a binding partner of the protein-capture agent of at least one patch on the
7 array; next, optionally washing said array to remove unbound or nonspecifically bound
8 components of the sample from the array; and in a final step, detecting, either directly or
9 indirectly, for the presence or amount of protein bound to each patch of the array.

10 In another embodiment of the invention, a method of assaying in parallel for a
11 plurality of different proteins in a sample which are expression products, or fragments
12 thereof, of a cell or a population of cells in an organism, comprises first delivering the
13 sample to the invention array of protein-capture agents under conditions suitable for protein
14 binding, wherein each of the proteins being assayed is a binding partner of the protein-
15 capture agent of at least one patch on the array. The first step may be followed by an
16 optional step of washing the array with fluid to remove unbound or nonspecifically bound
17 components of the sample from the array. Lastly, the presence or amount of protein bound
18 to each patch is detected, either directly or indirectly.

19 A variety of different embodiments of the invention array of protein-capture agents
20 may be used in the methods for assaying in parallel for a plurality of different proteins in a
21 sample which are expression products, or fragments thereof, of a cell or a population of cells
22 in an organism. Generally, preferred embodiments of these methods comprise the use of

1 preferred arrays of the invention. For instance, in preferred embodiments of the method, the
2 protein-capture agents are antibodies or antibody fragments. In further preferred
3 embodiments for assaying the different amounts of a plurality of proteins in a cell in parallel
4 or the protein expression pattern of a cell, the plurality of patches on the array can bind at
5 least about 100 or at least about 10^3 different proteins which are the expression products, or
6 fragments thereof, of a cell or population of cells in an organism. Alternatively, the plurality
7 of patches on the array used in the methods can bind at least about 10^4 different proteins
8 which are the expression products, or fragments thereof, of a cell or population of cells in an
9 organism.

10 The methods of assaying in parallel for a plurality of different proteins in a sample
11 which are expression products, or fragments thereof, of a cell or a population of cells in an
12 organism, optionally comprise the additional step of further characterizing the protein bound
13 to at least one patch of the array. This step is typically designed to identify the nature of the
14 protein bound to the protein-capture agent of a particular patch. In some cases, the entire
15 identity of the bound protein may not be known and the purpose of the further
16 characterization may be the initial identification of the mass, sequence, structure and/or
17 activity of the bound protein. In other cases, the basic identity of the protein may be known,
18 but the post-translational modification, activation state, or some other feature of the protein
19 may not be known. In one embodiment, the step of further characterizing the proteins
20 involves measuring the activity of the proteins. Although in some cases it may be preferable
21 to remove the protein from the patch before the step of further characterizing the protein is
22 carried out, in other cases the protein can be further characterized while still bound to the

1 patch. In still further embodiments, the protein-capture agents of the patch which binds a
2 protein can be used to isolate and/or purify the protein from cells. The purified sample can
3 then be characterized through traditional means such as microsequencing, mass
4 spectrometry, and the like.

5 In another embodiment, the present invention provides a method of determining the
6 protein expression pattern of a cell or population of cells in an organism. This method
7 involves first delivering a sample containing expression products, or fragments thereof, of
8 the cell or population of cells to the protein-capture agent array of the invention under
9 conditions suitable for protein binding. The presence and/or amount of protein bound to each
10 patch can then be determined by a suitable detection means. The detection may be either
11 direct or indirect. Quantitative detection is typically preferred for this application (and for
12 other proteomics applications). The method preferably further comprises an additional step
13 before the detection step comprising washing the array to remove unbound or
14 nonspecifically bound components of the sample from the array. The amount of protein
15 bound to a patch of the array may optionally be determined relative to the amount of a
16 second protein bound to a second patch of the array. The method of determining the protein
17 expression pattern of a cell or a population of cells in an organism, optionally comprises the
18 additional step of further characterizing the proteins bound to at least one patch of the array,
19 as previously described above.

20 In the method of assaying the protein expression pattern of a cell or population of
21 cells in an organism, many of the targets of the protein-capture agents of the array may
22 optionally be of unknown sequence, identity, and/or function. For instance, the antibodies of

1 the array may have been prepared by selecting a phage display library by affinity binding to
2 the immobilized proteins of a cellular extract which contains many unidentified proteins. If
3 the protein bound by a protein-capture agent on a particular patch of an array is unknown,
4 but is of interest, then that protein may optionally be later identified or characterized by first
5 using the same protein-capture agent that was used on the array to isolate the protein in
6 question from cells. The isolated binding partner from the cell can then be assayed directly
7 for function and/or sequenced.

8 The arrays of protein-capture agents may also be used to compare the protein
9 expression patterns of two cells or populations of cells. In this method, a sample containing
10 expression products, or fragments thereof, of a first cell or population of cells is delivered to
11 the invention array of protein-capture agents under conditions suitable for protein binding.
12 In an analogous manner, a sample containing expression products, or fragments thereof, of a
13 second cell or population of cells to a second array, is delivered to a second array which is
14 identical to the first array. Preferably, both arrays are then washed to remove unbound or
15 nonspecifically bound components of the sample from the arrays. In a final step, the
16 amounts of protein remaining bound to the patches of the first array are compared to the
17 amounts of protein remaining bound to the corresponding patches of the second array. If it
18 is desired to determine the differential protein expression pattern of two cells or populations
19 of cells, for instance, then the amount of protein bound to the patches of the first array may
20 be subtracted from the amount of protein bound to the corresponding patches of the second
21 array.

1 Methods of comparing the protein expression of two cells or populations of cells are
2 particularly useful for the understanding of biological processes. For instance, using these
3 methods, the protein expression patterns of identical cells or closely related cells exposed to
4 different conditions can be compared. Most typically, the protein content of one cell or
5 population of cells is compared to the protein content of a control cell or population of cells.
6 For instance, in one embodiment of the invention, one of the cells or populations of cells is
7 neoplastic and the other cell is not. In another embodiment, one of the two cells or
8 populations of cells being assayed is infected with a pathogen. Alternatively, one of the two
9 cells or populations of cells has been exposed to a stressor and the other cell or population of
10 cells serves as a control. The stressor may optionally be chemical, environmental, or
11 thermal. One of the two cells may optionally be exposed to a drug or a potential drug and its
12 protein expression pattern compared to a control cell.

13 Such methods of assaying differential gene expression at the protein level are useful
14 in the identification and validation of new potential drug targets as well as for drug
15 screening. For instance, the method may be used to identify a protein which is
16 overexpressed in tumor cells, but not in normal cells. This protein may be a target for drug
17 intervention. Inhibitors to the action of the overexpressed protein can then be developed.
18 Alternatively, antisense strategies to inhibit the overexpression may be developed. In
19 another instance, the protein expression pattern of a cell, or population of cells, which has
20 been exposed to a drug or potential drug can be compared to that of a cell, or population of
21 cells, which has not been exposed to the drug. This comparison will provide insight as to
22 whether or not the drug has had the desired effect on a target protein (drug efficacy) and

1 whether other proteins of the cell, or population of cells, have also been affected (drug
2 specificity).

3 The arrays of the present invention are also suitable for diagnostic applications and
4 suitable for use in diagnostic devices. The high density of the antibodies on some arrays of
5 the present invention enables a large number of different, antibody-based diagnostic tests to
6 be formatted onto a single biochip. The protein-capture agents on the invention array can be
7 used to evaluate the status of a disease condition in a tissue, such as a tumor, where the
8 expression levels of certain proteins in the cells of the tissue is known to be indicative of a
9 particular type of disease condition or stage of a disease condition. If certain patterns of
10 protein expression are not previously known to be indicative of a disease state, the protein-
11 capture agent arrays of the invention can then first be used to establish this information.

12 Accordingly, in one embodiment, the invention provides a method of evaluating a
13 disease condition in a tissue of an organism comprising first contacting the invention array
14 of protein-capture agents with a sample comprising the expression products, or fragments
15 thereof, of the cells of the tissue being evaluated, wherein the contacting occurs under
16 conditions suitable for protein binding and wherein the binding partners of a plurality of
17 protein-capture agents on the array include proteins which are expression products, or
18 fragment thereof, of the cells of the tissue and whose expression levels are indicative of the
19 disease condition. The method next comprises detecting, either directly or indirectly, for the
20 presence of protein to each patch. In a preferred embodiment, the method further comprises
21 the step of washing the array to remove unbound or nonspecifically bound components of
22 the sample from the array. In such a method, the array will typically comprise protein-

capture agents which bind those proteins whose presence, absence, or relative amount in cells is known to be indicative of a particular type of disease condition or state of a disease condition. For instance, the plurality of proteins being assayed in such a method may include such proteins as HER2 protein or prostate-specific antigen (PSA).

(h) Examples.

The following specific examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims:

Example 1. Fabrication of a two-dimensional array by photolithography.

In a preferred embodiment of the invention, two-dimensional arrays are fabricated onto the substrate material via standard photolithography and/or thin film deposition. Alternative techniques include microcontact printing. Usually, a computer-aided design pattern is transferred to a photomask using standard techniques, which is then used to transfer the pattern onto a silicon wafer coated with photoresist.

In a typical example, the array ("chip") with lateral dimensions of 10 x 10 mm comprises squared patches of a bioreactive layer (here: gold as the coating on a silicon substrate) each 0.1 x 0.1 mm in size and separated by hydrophobic surface areas with a 0.2 mm spacing. 4" diameter Si(100) wafers (Virginia Semiconductor) are used as bulk materials. Si(100) wafers are first cleaned in a 3:1 mixture of H₂SO₄, conc.: 30% H₂O₂ (90°C, 10 min), rinsed with deionized water (18 MΩcm), finally passivated in 1% aqueous HF, and singed at 150°C for 30 min to become hydrophobic. The wafer is then spincoated

1 with photoresist (Shipley 1813), prebaked for 25 minutes at 90°C, exposed using a Karl Suss
2 contact printer and developed according to standard protocols. The wafer is then dried and
3 postbaked at 110°C for 25 min. In the next step, the wafer is primed with a titanium layer of
4 20 nm thickness, followed by a 200 nm thick gold layer. Both layers were deposited using
5 electron-beam evaporation (5 Å/s). After resist stripping and a short plasma treatment, the
6 gold patches can be further chemically modified to achieve the desired bioreactive and
7 biocompatible properties (see Example 3, below).

8 Example 2. Fabrication of a two-dimensional array by deposition through a hole
9 mask.

10 In another preferred embodiment the array of gold patches is fabricated by thin film
11 deposition through a hole mask which is in direct contact with the substrate. In a typical
12 example, Si(100) wafers are first cleaned in a 3:1 mixture of H₂SO₄, conc.: 30% H₂O₂
13 (90°C, 10 min), rinsed with deionized water (18 MΩcm), finally passivated in 1% aqueous
14 HF and singed at 150°C for 30 min to become hydrophobic. The wafer is then brought into
15 contact with a hole mask exhibiting the positive pattern of the desired patch array. In the
16 next step, the wafer is primed with a titanium layer of 20 nm thickness, followed by a 200
17 nm thick gold layer. Both layers were deposited using electron-beam evaporation (5 Å/s).
18 After removal of the mask, the gold patches can be further chemically modified to achieve
19 the desired bioreactive and biocompatible properties (see Example 3, below).

20 Example 3. Synthesis of an aminoreactive monolayer molecule (following the
21 procedure outlined in Wagner *et al.*, *Biophys. J.*, 1996, 70:2052-2066).

1 *General.* ^1H - and ^{13}C -NMR spectra are recorded on Bruker instruments (100 to 400
2 MHz). Chemical shifts (δ) are reported in ppm relative to internal standard $((\text{CH}_3)_4\text{Si}$, δ =
3 0.00 (^1H - and ^{13}C -NMR)). FAB-mass spectra are recorded on a VG-SABSEQ instrument
4 (Cs^+ , 20 keV). Transmission infrared spectra are obtained as dispersions in KBr on an FTIR
5 Perkin-Elmer 1600 Series instrument. Thin-layer chromatography (TLC) is performed on
6 precoated silica gel 60 F254 plates (MERCK, Darmstadt, FRG), and detection was done
7 using Cl_2 /toluidine, PdCl_2 and UV-detection under NH_3 -vapor. Medium pressure liquid
8 chromatography (MPLC) is performed on a Labomatic MD-80 (LABOMATIC INSTR. AG,
9 Allschwil, Switzerland) using a Buechi column (460x36 mm; BUECHI, Flawil,
10 Switzerland), filled with silica gel 60 (particle size 15-40 μm) from Merck.

11 *Synthesis of 11,11'-dithiobis(succinimidylundecanoate) (DSU).* Sodium thiosulfate
12 (55.3 g, 350 mmol) is added to a suspension of 11-bromo-undecanoic acid (92.8 g, 350
13 mmol) in 50% aqueous 1,4-dioxane (1000 ml). The mixture is heated at reflux (90°C) for 2
14 h until the reaction to the intermediate Bunte salt was complete (clear solution). The
15 oxidation to the corresponding disulfide is carried out *in situ* by adding iodine in portions
16 until the solution retained with a yellow to brown colour. The surplus of iodine is retitrated
17 with 15% sodium pyrosulfite in water. After removal of 1,4-dioxane by rotary evaporation
18 the creamy suspension is filtered to yield product *11,11'-dithiobis(undecanoic acid)*.
19 Recrystallization from ethyl acetate/THF provides a white solid (73.4 g, 96.5%): mp 94°C;
20 ^1H NMR (400 MHz, CDCl_3 / CD_3OD 95 : 5): δ 2.69 (t, 2H, J = 7.3 Hz), 2.29 (t, 2H, J = 7.5
21 Hz), 1.76-1.57 (m, 4H), and 1.40-1.29 (m, 12H); FAB-MS (Cs^+ , 20 keV): m/z (relative

intensity) 434 (100, M^+). Anal. Calcd. for $C_{22}H_{42}O_4S_2$: C, 60.79; H, 9.74; S, 14.75. Found: C, 60.95; H, 9.82; S, 14.74. To a solution of *11,11'*-dithiobis(*undecanoic acid*) (1.0 g, 2.3 mmol) in THF (50 ml) is added N-hydroxysuccinimide (0.575 g, 5 mmol) followed by DCC (1.03 g, 5 mmol) at 0°C. After the reaction mixture is allowed to warm to 23°C and is stirred for 36 h at room temperature, the dicyclohexylurea (DCU) is filtered. Removal of the solvent under reduced pressure and recrystallization from acetone/hexane provides *11,11'*-dithiobis(succinimidylundecanoate) as a white solid. Final purification is achieved by medium pressure liquid chromatography (9 bar) using silica gel and a 2:1 mixture of ethyl acetate and hexane. The organic phase is concentrated and dried in vacuum to afford *11,11'*-dithiobis(succinimidylundecanoate) (1.12 g, 78%): mp 95°C; 1H NMR (400 MHz, $CDCl_3$): δ 2.83 (s, 4H), 2.68 (t, 2H, $J = 7.3$ Hz), 2.60 (t, 2H, $J = 7.5$ Hz), 1.78-1.63 (m, 4H), and 1.43-1.29 (m, 12H); FAB-MS (Cs^+ , 20 keV): m/z (relative intensity) 514 (100), 628 (86, M^+). Anal. Calcd. for $C_{30}H_{48}N_2O_8S_2$: C, 57.30; H, 7.69; N, 4.45; S, 10.20. Found: C, 57.32; H, 7.60; N, 4.39; S, 10.25.

Example 4. Formation of an aminoreactive monolayer on gold (following the procedure of Wagner *et al.*, *Biophys. J.*, 1996, 70:2052-2066).

Monolayers based on *11,11'*-dithiobis(succinimidylundecanoate) (DSU) can be deposited on Au(111) surfaces of substrates described under Examples 1 and 2 by immersing them into a 1 mM solution of DSU in chloroform at room temperature for 1 hour. After rinsing with 10 volumes of solvent, the N-hydroxysuccinimidyl-terminated monolayer

1 is dried under a stream of nitrogen and immediately used for immobilization of the protein-
2 capture agents.

3
4 Example 5. Formation and use of an array of immobilized Fab' antibody fragments
5 to detect concentrations of soluble proteins prepared from cultured mammalian cells.

6 Collections of IgG antibodies are purchased from commercial sources (e.g. Pierce,
7 Rockford, IL). The antibodies are first purified by affinity chromatography based on binding
8 to immobilized protein A. The antibodies are diluted 1:1 in binding buffer(0.1 M Tris-HCl,
9 0.15 M NaCl, pH 7.5). A 2 ml minicolumn containing a gel with immobilized protein A is
10 prepared. (Hermanson, *et. al.*, *Immobilized Affinity Ligand Techniques*, Academic Press,
11 San Diego, 1992.) The column is equilibrated with 10 ml of binding buffer. Less than 10 mg
12 of immunoglobulin is applied to each 2 ml minicolumn and the column is washed with
13 binding buffer until the absorbance at 280 nm is less than 0.02. The bound immunoglobulins
14 are eluted with 0.1 M glycine, 0.15 M NaCl, pH 2.8, and immediately neutralized with 1.0
15 M Tris-HCl, pH 8.0 to 50 mM final concentration and then dialyzed against 10 mM sodium
16 phosphate, 0.15 M NaCl, pH 7.2 and stored at 4°C.

17 The purified immunoglobulin are digested with immobilized pepsin. Pepsin is an
18 acidic endopeptidase and hydrolyzes proteins favorably adjacent to aromatic and
19 dicarboxylic L-amino acid residues. Digestion of IgG with pepsin generates intact F(ab')₂
20 fragments. Immobilized pepsin gel is washed with digestion buffer; 20 mM sodium acetate,
21 pH 4.5. A solution of purified IgG at 10 mg/ml is added to the immobilized pepsin gel and
22 incubated at 37°C for 2 hours. The reaction is neutralized by the addition of 10 mM Tris-

1 HCl, pH 7.5 and centrifuged to pellet the gel. The supernatant liquid is collected and applied
2 to an immobilized protein A column, as described above, to separate the F(ab')₂ fragments
3 from the Fc and undigested IgG. The pooled F(ab')₂ is dialyzed against 10 mM sodium
4 phosphate, 0.15 M NaCl, pH 7.2 and stored at 4°C. The quantity of pooled, eluted F(ab')₂ is
5 measured by peak area absorbance at 280 nm.

6 The purified F(ab')₂ fragments at a concentration of 10 mg/ml are reduced at 37 °C
7 for 1 hour in a buffer of 10 mM sodium phosphate, 0.15 M NaCl, 10 mM 2-
8 mercaptoethylamine, 5 mM EDTA, pH 6.0. The Fab' fragments are separated from unsplit
9 F(ab')₂ fragments and concentrated by application to a Sephadex G-25 column (M_r = 46,000
10 – 58,000). The pooled Fab' fragments are dialyzed against 10 mM sodium phosphate, 0.15
11 M NaCl, pH 7.2. The reduced Fab' fragments are diluted to 100 µg/ml and applied onto the
12 bioreactive patches containing exposed aminoreactive functional groups using a computer-
13 aided, capillary-based microdispensing system (for antibody immobilization procedures, see
14 Dammer *et al.*, *Biophys. J.*, **70**:2437-2441, 1996). After an immobilization period of 30
15 minutes at 30°C, the array is rinsed extensively with 10 mM sodium phosphate, 0.15 M
16 NaCl, 5 mM EDTA, pH 7.0.

17 Transformed human cells grown in culture are collected by low speed centrifugation,
18 briefly washed with ice-cold phosphate-buffered solution (PBS), and then resuspended in
19 ice-cold hypotonic buffer containing DNase/RNase (10 µg/ml each, final concentration) and
20 a mixture of protease inhibitors. Cells are transferred to a microcentrifuge tube, allowed to
21 swell for 5 minutes, and lysed by rapid freezing in liquid nitrogen and thawing in ice-cold
22 water. Cell debris and precipitates are removed by high-speed centrifugation and the

1 supernatant is cleared by passage through a 0.45 μ m filter. The cleared lysate is applied to
2 the Fab' fragment array described above and allowed to incubate for 2 hours at 30°C. After
3 binding the array is washed extensively with 10 mM sodium phosphate, 0.15 M NaCl, 5 mM
4 EDTA, pH 7.0. The location and amount of bound proteins are determined by optical
5 detection.

6 Example 6. Formation and use of an array of immobilized antibody fragments to
7 detect concentrations of soluble proteins prepared from cultured mammalian cells.

8 A combinatorial library of filamentous phage expressing scFv antibody fragments is
9 generated based on the technique of McCafferty and coworkers; McCafferty, *et al.*, *Nature*,
10 1990, **348**:552-554; Winter and Milstein, *Nature*, 1991, **349**:293-299. Briefly, mRNA is
11 purified from mouse spleens and used to construct a cDNA library. PCR fragments encoding
12 sequences of the variable heavy and light chain immunoglobulin genes of the mouse are
13 amplified from the prepared cDNA. The amplified PCR products are joined by a linker
14 region of DNA encoding the 15 amino acid peptide (Gly₄SerGly₂CysGlySerGly₄Ser) (SEQ
15 ID NO: 1) and the resulting full-length PCR fragment is cloned into an expression plasmid
16 (pCANTAB 5 E) in which the purification peptide tag (E Tag) has been replaced by a His₆
17 peptide (SEQ ID NO: 2). Electrocompetent TG1 *E.coli* cells are transformed with the
18 expression plasmid by electroporation. The pCANTAB-transformed cells are induced to
19 produced functional filamentous phage expressing scFv fragments by superinfection with
20 M13KO7 helper phage. Cells are grown on glucose-deficient medium containing the
21 antibiotics ampicillin (to select for cells with the phagemid) and kanamycin (to select for

1 cells infected with M13KO7). In the absence of glucose, the lac promoter present on the
2 phagemid is no longer repressed, and synthesis of the scFv-gene 3 fusion begins.

3 Proteins from a cell lysate are adsorbed to the wells of a 96-well plate. Transformed
4 human cells grown in culture are collected by low speed centrifugation and the cells are
5 briefly washed with ice-cold PBS. The washed cells are then resuspended in ice-cold
6 hypotonic buffer containing DNase/RNase (10 µg/ml each, final concentration) and a
7 mixture of protease inhibitors, allowed to swell for 5 minutes, and lysed by rapid freezing in
8 liquid nitrogen and thawing in ice-cold water. Cell debris and precipitates are removed by
9 high-speed centrifugation and the supernatant is cleared by passage through a 0.45 µm filter.
10 The cleared lysate is diluted to 10 µg/ml in dilution buffer; 20 mM PIPES, 0.15 M NaCl, 0.1
11 % CHAPS, 10%, 5 mM EDTA, 5 mM 2-mercaptoethanol, 2 mM DTT, pH 7.2 and applied
12 to the 96-plate wells. After immobilization for 1 hour at 30°C, the well is washed with the
13 dilution buffer and then incubated with dilution buffer containing 10% nonfat dry milk to
14 block unreacted sites. After the blocking step, the well is washed extensively with the
15 dilution buffer.

16 Phage expressing displayed antibodies are separated from *E. coli* cells by
17 centrifugation and then precipitated from the supernatant by the addition of 15% w/v PEG
18 8000, 2.5 M NaCl followed by centrifugation. The purified phage are resuspended in the
19 dilution buffer containing 3% nonfat dry milk and applied to the well containing the
20 immobilized proteins described above, and allowed to bind for 2 hours at 37°C, followed by
21 extensive washing with the binding buffer. Phage are eluted from the well with an elution
22 buffer; 20 mM PIPES, 1 M NaCl, 0.1 % CHAPS, 10%, 5 mM EDTA, 5 mM 2-

1 mercaptoethanol, 2 mM DTT, pH 7.2. The well is then extensively washed with purge
2 buffer; 20 mM PIPES, 2.5 M NaCl, 0.1 % CHAPS, 10%, 5 mM EDTA, 5 mM 2-
3 mercaptoethanol, 2 mM DTT, pH 7.2. The well is then extensively washed with dilution
4 buffer; 20 mM PIPES, 0.15 M NaCl, 0.1 % CHAPS, 10%, 5 mM EDTA, 5 mM 2-
5 mercaptoethanol, 2 mM DTT, pH 7.2. The eluted phage solution is then re-applied to a new
6 well containing adsorbed antigen and the panning enrichment is repeated 4 times. Finally,
7 the phage are eluted from the well with 2M of NaCl in 20 mM PIPES, 0.1 % CHAPS, 10%,
8 5 mM EDTA, 5 mM 2-mercaptoethanol, 2 mM DTT, pH 7.2. Eluates are collected and
9 mixed with log-phase TG1 cells, and grown at 37°C for 1 hour and then plated onto SOB
10 medium containing ampicillin and glucose and allowed to grow for 12 – 24 hours.

11 Individual colonies are picked and arrayed into 96-well 2ml blocks containing SOB
12 medium and M13KO7 helper phage and grown for 8 hours with shaking at 37°C. The phage
13 are separated from cells by centrifugation and precipitated with PEG/NaCl as described
14 above. Concentrated phage are used to infect HB2151 *E. coli*. *E. coli* TG1 produces a
15 suppressor tRNA which allows readthrough (suppression) of an amber stop codon located
16 between the scFv and phage gene 3 sequences of the pCANTAB 5 E plasmid. Infected
17 HB2151 cells are selected on medium containing ampicillin, glucose, and nalidixic acid.
18 Cells are grown to mid-log and then centrifuged and resuspended in medium lacking glucose
19 and growth continued. Soluble scFv fragments will accumulate in the cell periplasm. A
20 periplasmic extract is prepared from pelleted cells by mild osmotic shock. The soluble scFv
21 released into the supernatant is purified by affinity binding to Ni-NTA activated agarose and
22 eluted with 10 mM EDTA.

1 The purified scFv antibody fragments are diluted to 100 µg/ml and applied onto the
2 bioreactive patches with exposed aminoreactive groups using a computer-aided, capillary-
3 based microdispensing system. After an immobilization period of 30 minutes at 30°C, the
4 array is rinsed extensively with 10 mM sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH
5 7.0.

6 Transformed human cells grown in culture are collected by low speed centrifugation,
7 briefly washed with ice-cold PBS, and then resuspended in ice-cold hypotonic buffer
8 containing DNase/RNase (10 µg/ml each, final concentration) and mixture of protease
9 inhibitors. Cells are transferred to a microcentrifuge tube, allowed to swell for 5 minutes,
10 and lysed by rapid freezing in liquid nitrogen and thawing in ice-cold water. Cell debris and
11 precipitates are removed by high-speed centrifugation and the supernatant is cleared by
12 passage through a 0.45 µm filter. The cleared lysate is applied to the scFv fragment array
13 described above and allowed to incubate for 2 hours at 30°C. After binding, the array is
14 washed extensively with 0.1 M sodium phosphate, 0.15 M NaCl, 5 mM EDTA pH 7.0. The
15 location and amount of bound proteins are determined by optical detection.

1 Patterns of binding are established empirically by testing dilutions of a control cell
2 extract. Extracts from experimental cells are diluted to a series of concentrations and then
3 tested against the array. Patterns of protein expression in the experimental cell lysates are
4 compared to protein expression patterns in the control samples to identify proteins with
5 unique expression profiles.

6
7 Example 7. Formation and use of an array of immobilized monoclonal antibodies
8 to detect concentrations of soluble proteins prepared from cultured mammalian cells.

9 Collections of monoclonal antibodies are purchased from commercial suppliers as
10 either raw ascities fluid or purified by chromatography over protein A, protein G, or protein
11 L. If from raw ascites fluid, the antibodies are purified using a HiTrap Protein G or HiTrap
12 Protein A column (Pharmacia) as appropriate for the immunoglobulin subclass and species.
13 Prior to chromatography the ascites are diluted with an equal volume of 10 mM sodium
14 phosphate, 0.9 % NaCl, pH 7.4 (PBS) and clarified by passage through a 0.22 μ m filter. The
15 filtrate is loaded onto the column in PBS and the column is washed with two column
16 volumes of PBS. The antibody is eluted with 100 mM Glycine-HCl, pH 2.7 (for protein G)
17 or 100 mM citric acid, pH 3.0 (for protein A). The eluate is collected into 1/10 volume 1 M
18 Tris-HCl, pH 8.0. The final pH is 7.5. Fractions containing the antibodies are confirmed by
19 SDS-PAGE and then pooled and dialyzed against PBS.

20 The different samples of purified antibodies are each diluted to 100 μ g/ml. Each
21 different antibody sample is applied to a separate patch of an array of aminoreactive
22 monolayer patches (see Example 4, above) using a computer-aided, capillary-based

1 microdispensing system. After an immobilization period of 30 minutes at 30°C, the array is
2 rinsed extensively with 10 mM sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 7.0.

3 Transformed human cells grown in culture are collected by low speed centrifugation,
4 briefly washed with ice-cold PBS, and resuspended in ice-cold hypotonic buffer containing
5 Dnase/Rnase (10 µg/ml each, final concentration) and a mixture of protease inhibitors. Cells
6 are transferred to a microcentrifuge tube, allowed to swell for 5 minutes, and lysed by rapid
7 freezing in liquid nitrogen and thawing in ice-cold water. Cell debris and precipitates are
8 removed by high-speed centrifugation and the supernatant is cleared by passage through a
9 0.45 µm filter. The cleared lysate is applied to the monoclonal antibody array described
10 above and allowed to incubate for 2 hours at 30°C. After binding the array is washed
11 extensively as in Example 6, above. The location and amount of bound proteins are
12 determined by optical detection.

13
14 All documents cited in the above specification are herein incorporated by reference.
15 In addition, the copending U.S. patent application "Arrays of Proteins and Methods of Use
16 Thereof", filed on July 14, 1999, with the identifier 24406-0004 P1, for the inventors Peter
17 Wagner, Dana Ault-Riche, Steffen Nock, and Christian Itin, is herein incorporated by
18 reference in its entirety. Various modifications and variations of the present invention will
19 be apparent to those skilled in the art without departing from the scope and spirit of the
20 invention. Although the invention has been described in connection with specific preferred
21 embodiments, it should be understood that the invention as claimed should not be unduly
22 limited to such specific embodiments. Indeed, various modifications of the described modes

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1 for carrying out the invention which are obvious to those skilled in the art are intended to be
2 within the scope of the following claims.

3

[illegible]